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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Our earlier studies demonstrated that ibuprofen sensitizes prostate cancer cells to radiation *in vitro* and *in vivo*. The cytotoxic and radiosensitizing effects of NSAIDs were seen at concentrations that are higher than those that inhibit prostaglandin synthesis. To understand the molecular mechanisms involved in radiosensitization we examined the effects of ibuprofen on several potential cellular targets including COX-2 and transcription factors NFkB and HIFs. NFkB regulates cytokines, growth factors and controls apoptotic pathways. Ibuprofen inhibited constitutive as well as cytokine- or radiation-induced NFkB. Tumor cells in hypoxic environment are resistant to cancer therapies including radiation, although the role of HIFs in this effect is not clear. HIFs are key regulators of tumor growth and angiogenesis, and are over expressed in the majority of common human tumors. Since NSAIDs inhibit angiogenesis we determined the effect of the NSAIDs on HIFs. Ibuprofen treatment rapidly reduced HIF proteins with a subsequent reduction in the HIF-regulated gene products VEGF and Glut-1 under hypoxia *in vitro*. We are currently evaluating the effects of radiation in combination with COX-2 specific inhibitors that appear to be relatively less toxic in clinic, on tumor growth and angiogenesis *in vitro* and *in vivo*. Preclinical studies using a combination of COX inhibitors is in progress in preparation for a clinical trial with "molecular markers". The specific role of HIFs is being evaluated in HIF over expressing cells as another therapeutic target for radiosensitization. The results from this research project can be directly translated into the clinic with a potential to improve local tumor control, to reduce toxicity and increase overall survival.

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Introduction:

Radiation therapy is a mainstay of local-regional treatment of prostate cancer although the efficacy is limited for larger sized tumors. To complement the physical advances in radiation dose delivery, novel biological approaches are necessary to increase tumor cell killing and decrease normal tissue injury. Our novel approach of using non-steroidal anti-inflammatory drugs (NSAIDs) to enhance the effects of radiation therapy was based on a clinical observation that the NSAID ibuprofen ameliorated acute radiation-induced urinary symptoms. Although ibuprofen appeared to have a potential radioprotective activity against the acute effects of treatment, some reports in the literature suggested that NSAIDs were useful in cancer treatment, either alone or in combination with radiation therapy. To investigate the role of NSAIDs as potential radiation modifiers for prostate cancer treatment, we conducted *in vitro* and *in vivo* experiments (1, 2). These studies showed that ibuprofen enhanced radiation response in both tissue culture and animals. Furthermore, the response *in vivo* was found to be superior to that of anti-androgens (2).

The accepted hypothesis for mechanisms underlying the actions of NSAIDs is that NSAIDs inhibit cyclooxygenase enzymes thereby inhibiting prostaglandin (PG) synthesis. There are two isoforms of cyclooxygenase, COX-1 and COX-2, and nonspecific NSAIDs inhibit both isoforms. COX-1 is constitutively expressed in most tissues and produces prostaglandins that are essential for homeostasis of platelets, kidney and gut mucosa. Inhibition of COX-1 therefore results in undesirable toxicity in the patients. COX-2 is an inducible enzyme, induced in response to stress stimuli including inflammatory cytokines, growth factors and hypoxia. Several reports indicate that COX-2 is constitutively expressed in a variety of human tumors (3). Although the COX isoforms are inhibited by NSAIDs at concentrations in submicromolar range (4), in vitro the cytotoxic effects of NSAIDs including induction of apoptosis are seen at much higher doses (1, 5-10). Therefore, it appears that mechanisms in addition to, or other than the inhibition prostaglandin synthesis are involved in growth inhibition and anti-tumor effects of NSAIDs and that NSAIDs have multiple cellular targets.

As proposed in our grant application, we investigated the effects of NSAIDs on two potential non COX-2 molecular targets. 1) NFkB, the key transcription factor in cytokine regulation, that also has anti-apoptotic properties, and 2) HIFs, the hypoxia inducible transcription factors HIF-1α and HIF-2α, which in hypoxic tumor environment regulate tumor angiogenesis by up regulating vascular endothelial growth factor (VEGF), VEGF receptor and other proangiogenic factors. Moreover, keeping up to date with the recent developments in COX-2 field, studies on COX-2 have been continued. COX-2 specific inhibitors appear to be particularly suitable to use in the clinic, as they are less toxic than the nonspecific NSAIDs. COX-2 specific inhibitors also reportedly inhibit tumor angiogenesis and thus may be of significant importance in cancer therapy. The majority of this work is done *in vitro* using 2 human prostate carcinoma cell lines, PC3 and DU-145. Whereas PC3 cells expressed COX-2 constitutively, DU-145 cells did not express the protein by western analysis.

1. Effect of NSAIDs on COX :

a) NSAIDs inhibit prostaglandin synthesis:

Effect of NSAIDs on prostaglandin synthesis in PC3 cells was measured using a PGE2 ELISA kit (Oxford Biochemical Research). Cells were plated in 6-well plates and prostaglandin synthesis was stimulated by adding 30 μM arachidonic acid. NSAIDs inhibited PGE_2 synthesis at low micromolar concentrations (<10 μM) (Appendix, *Figure 1*).

b) Effect of NSAIDs on COX-2 protein levels:
COX-2 protein expression in PC3, DU-145 and LNCaP cells was studied by western blot analysis. Whereas PC3 cells expressed COX-2 constitutively, DU-145 and LNCaP cells did not. Since the cytotoxic effects of NSAIDs were observed at higher concentrations, the effect of NSAIDs on COX-2 protein was studied by western blot analysis at those concentrations. In addition to the non-specific NSAIDs ibuprofen and diclofenac, we also studied the effect of COX-2 specific inhibitors NS 398, niflumic acid and nimesulide, and 5-lipoxygenase inhibitors MK886 and Rev 5901 on COX-2 protein levels in prostate cancer cells. Interestingly, COX-2 protein level *increased* when cells were treated with non-specific NSAIDs, COX-2 specific inhibitor NS-398 as well as lipoxygenase inhibitors (Appendix, *Figure 2*). The increase was evident at 6h and persisted up to 48h.

c) Effect of NSAIDs on COX-2 protein levels in combination with radiation or hypoxia:
Radiation: Radiation (8-10Gy) had no effect on COX-2 protein level in PC3 cells. However, ibuprofen-induced increase in COX-2 in irradiated cells was of a lesser magnitude as compared to the ibuprofen-induced increase in cells that were not irradiated (Fig. 1).

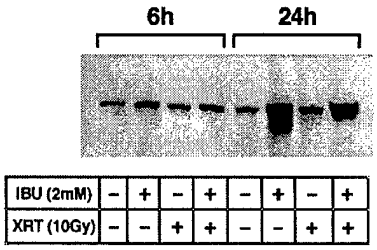
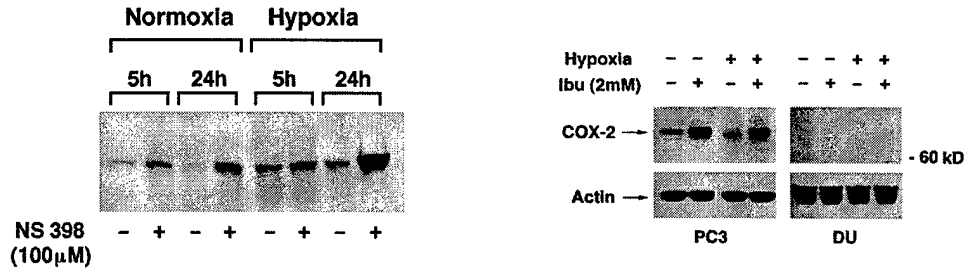


Fig. 1: COX-2 protein levels in PC3 cells treated with ibuprofen and radiation. PC3 cells constitutively express COX-2 protein. COX-2 levels significantly *increased* following ibuprofen treatment (2mM) in non-irradiated and irradiation cells.

Hypoxia: Hypoxia-induced increase in COX-2 protein was seen only if cells were grown in low serum media. Hypoxia did not change COX-2 protein level in PC3 cells grown in complete serum media. Ibuprofen and NS398, a COX-2 specific inhibitor, increased COX-2 protein levels under normoxic as well as hypoxic conditions (Fig. 2 A, B). DU-145 cells did not express COX-2 protein under normoxic condition nor was it induced in these cells by hypoxia or ibuprofen (Fig. 2B).



(A) (B)

Fig. 2

Fig. 2: (A) COX-2 protein levels in PC3 cells treated with NS398 (100 μM), under normoxic and hypoxic conditions. NS398 treatment was in 0.1% media. Hypoxia increased COX-2 protein. (B) Effect of ibuprofen on COX-2 protein in PC3 and DU-145 cells under normoxic and hypoxic conditions COX-2 levels *increased* significantly in cells treated with Ibuprofen and NS398 under normoxic and hypoxic conditions. Membrane was reprobed for actin as a loading control.

2. Radiosensitization of PC3 and DU-145 cells by COX-2 inhibitors and lipoxygenase inhibitors:

Our earlier studies indicated that ibuprofen enhanced radiation cytotoxicity in PC3 and DU-145 cells (1). As mentioned earlier, PC3 cells express COX-2 constitutively, whereas DU-145 cells do not express COX-2. Warner et al recently published a detailed analysis of COX-1/2 selectivities of >40 NSAIDs (4). As shown in Fig. 3, we further studied the effect of a number of NSAIDs characterized in that study and also 2 5-lipoxygenase inhibitors on clononogenic survival in PC3 cells in combination with radiation. 5-lipoxygenase inhibitors were cytotoxic at micromolar concentrations and also enhanced radiation cytotoxicity. However, COX-2 inhibitors were less cytotoxic and did not enhance radiation cytotoxicity at the concentrations used in these experiments (Fig. 3).

We have tested radiosensitizing potential of COX-2 specific inhibitors DFP, NS398, nimesulide, celecoxib and niflumic acid and dual inhibitors ketorolac, ibuprofen and diclofenac over abroad range of concentrations. Non-specific NSAID diclofenac was an effective radiosensitizer in both PC3 as well as DU-145 cells (Fig. 4). COX-2 specific inhibitors NS398 (100-300μM) and Celecoxib (40-80μM) increased cytotoxicity at higher concentrations but did not enhance radiosensitivity in either PC3 cells that expressed COX-2 or DU-145 cells that did not express COX-2 (Fig. 5 A, B). DFP (20-100μM) and ketorolac (0.5-2mM), were neither cytotoxic nor did they enhance radiosensitivity of PC3 and DU-145 cells (data not shown). These studies indicated that COX-2 might not have a role in radiosensitization by NSAIDs *in vitro*.

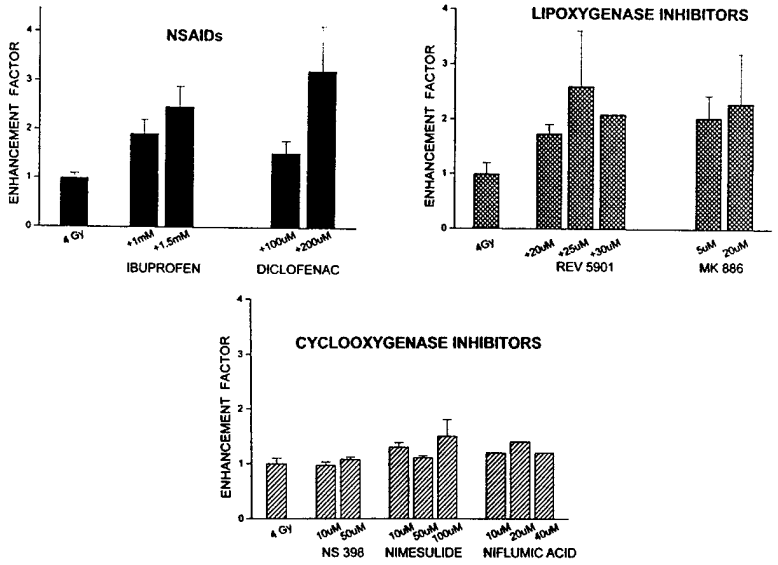


Fig. 3: PC3 cells were treated with various eicosanoid inhibitors, irradiated (4 Gy) and plated for clonogenic cell survival assay after 24h. NSAIDs (ibuprofen and diclofenac), and 5-lipoxygenase inhibitors (REV 5901 and MK886) enhanced radiation-induced cytotoxicity. COX-2 inhibitors (NS398, nimesulide and niflumaic acid) were not as effective at the concentrations used in this study.

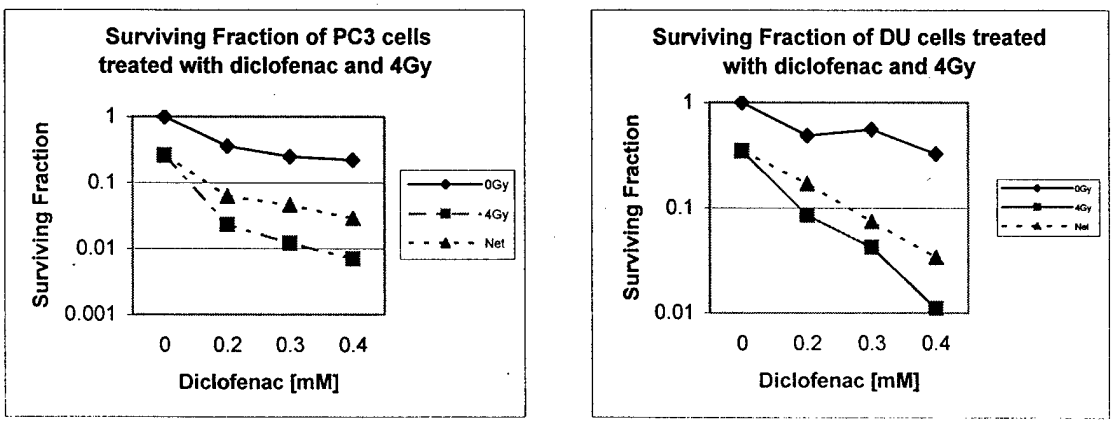
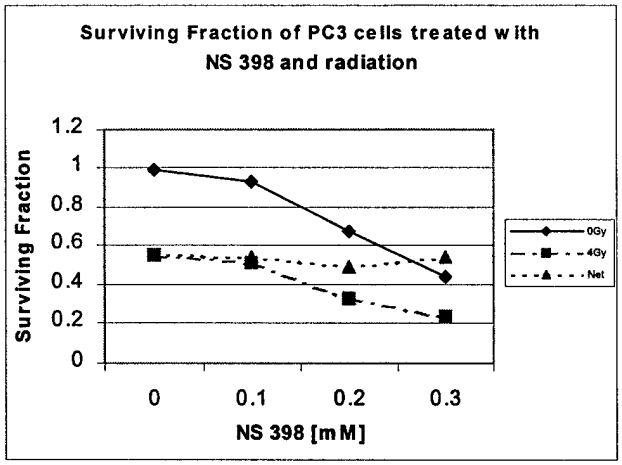


Fig. 4: PC3 and DU-145 cells were treated with different concentrations of diclofenac for 24 hours and irradiated. Cells were trypsinized and plated for clonogenic survival assay. Net survival was calculated by dividing the plating efficiency of combination group by the plating efficiency of cells treated with diclofenac alone.



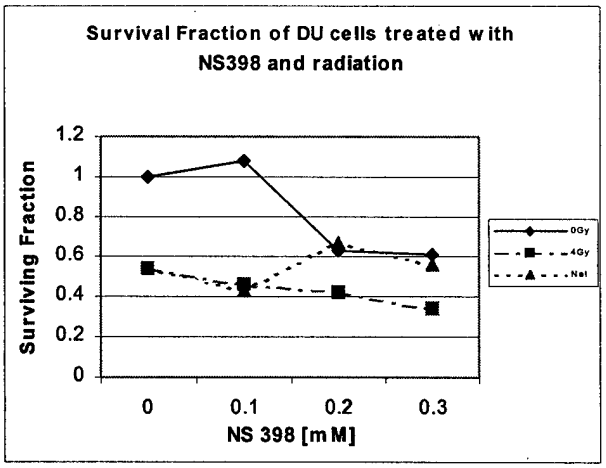
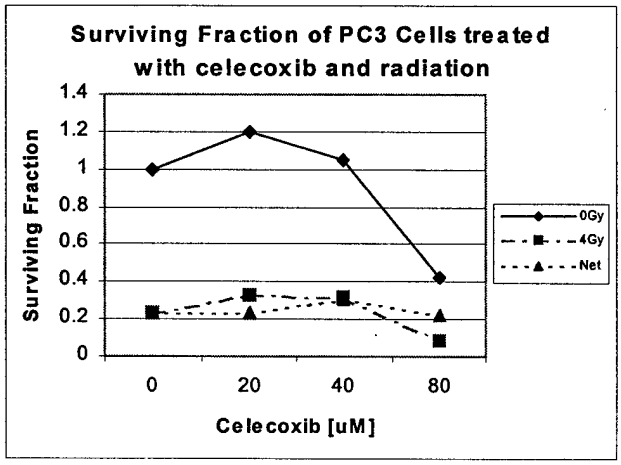


Fig. 5A: PC3 and DU-145 cells were treated with different concentrations of NS 398 for 2 h and irradiated. After 24 h cells were trypsinized and plated for clonogenic survival assay. Net survival was calculated by dividing the plating efficiency of combination group by the plating efficiency of cells treated with NS 398 alone.



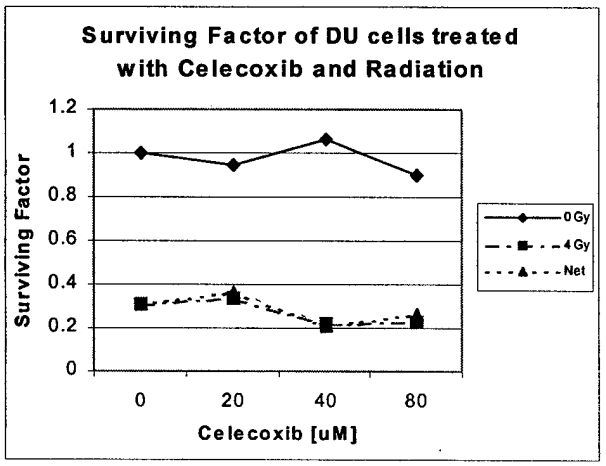


Fig. 5B: PC3 and DU-145 cells were treated with celecoxib for 24 h and irradiated. Cells were trypsinized and plated for clonogenic survival assay. Net survival was calculated by dividing the plating efficiency of combination group by the plating efficiency of cells treated with celecoxib alone.

3. Ibuprofen inhibited transcription factor NFkB :

Early on in our investigation, we postulated that the radiation enhancement effect of NSAIDs might be due to suppression of IL-6 (11), a growth promoting cytokine or possibly due to an increase in TNF-α, which had been shown to be a radiation sensitizer (12). Ibuprofen inhibited IL-6 and recombinant human IL-6 partially reversed the clonogenic radiosensitization of ibuprofen (Appendix, ***Table 1***). Ibuprofen increased TNF-α levels to some extent in irradiated cells but TNF did not enhance ibu-mediated radiosensitization (Appendix ***Table 1, Figure 3***). Cytokines are regulated by the transcription factor NF-kB. In addition to cytokine regulation, apoptotic pathways controlled by the Rel/NF-kB family of transcription factors appear to regulate the response of cells to DNA damage (13). Since ibuprofen modulated cytokine levels and induced apoptosis in PC3 cells, we examined the NF-kB status in several prostate cancer cell lines (Fig. 6, top panels). In the androgen-independent PC3 and DU-145 cells, the DNA-binding activity of NF-kB was constitutively activated. In contrast, the androgen–sensitive LNCaP cells had low level of NF-kB which was upregulated following exposure to cytokines or DNA damage. We next analyzed the activity of IkB-α kinase, IKK- α, which phosphorylates IkB- α and activates NF-kB. In PC3 cells, IKK- α activity was constitutively active, whereas LNCaP cells had minimal IKK- α activity that was activated by TNF (Fig. 6, bottom panels). We found that ibuprofen inhibited the constitutive activation of NF-kB and IKK- α in PC3 cells and blocked stimulated activation of NF-kB and IKK- α in LNCaP cells (14). The constitutive activation of NF-kB in prostate cancer cells may increase expression of growth promoting cytokines like IL6 as well as antiapoptotic proteins, thereby decreasing the effectiveness of anti-tumor therapy and contributing to the development of the malignant phenotype.

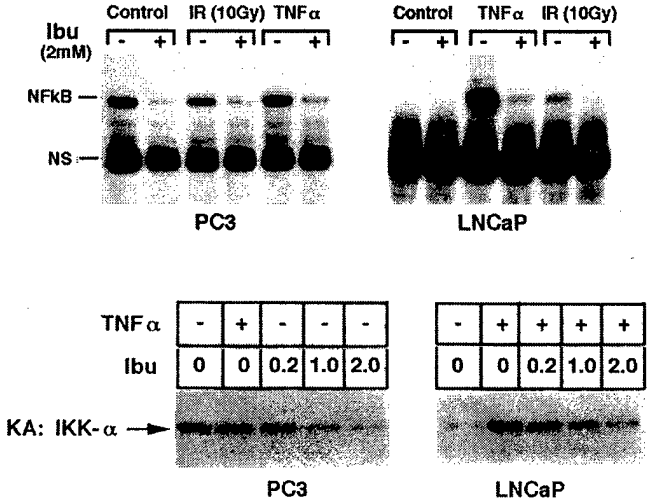


Fig. 6: Ibuprofen inhibited the constitutive and inducible NFκB and IKK-α kinase activity in prostate cancer cells. Top panels: Gel shift analysis of inhibition of NFκB DNA binding activity by ibuprofen in PC3 and LNCaP cells. Lower panels: Cells were incubated in the absence (-) and presence (+) of TNF for 15 min with indicated concentrations of ibuprofen. IKK-α kinase activity (KA: IKK-α).

4. HIFs (Hypoxia-inducible factors HIF-1α and HIF-2α) as molecular targets of NSAIDs:

Development of new blood vessels is a critical requirement for tumor growth (15, 16,). It is well known that tumor cells produce a variety of angiogenic factors including prostaglandins, basic fibroblast growth factor (bFGF), transforming growth factor-B (TGF-B) and vascular endothelial growth factor (VEGF). Although NSAIDs have been shown to inhibit angiogenesis, the specific mechanism responsible for the antiangiogenic actions of NSAIDs has not been defined. A number of studies have suggested that the anti-angiogenic effect of NSAIDs can be attributed to the specific inhibition of the COX-2 enzyme (17-21). However, even the tumor cells that lack COX-1 and COX-2 produce proangiogenic factors and stimulate angiogenesis (18) suggesting that in addition to COX-2, NSAIDs may target other angiogenic pathways as well (18, 22, 23). A major initiator of angiogenesis is hypoxia, which induces a wide variety of genes through the activation of transcription factor HIF-1α and HIF-2α, of the bHLH-PAS gene family (24-26). Under normoxic conditions HIFs interacts with tumor suppressor pVHL and are rapidly degraded via ubiquitin-dependent proteasome pathway (27-31). Hypoxia induces a rapid redox-sensitive increase in HIF protein stability and transcriptional activity (32-34) resulting in the activation of target genes involved in erythropoiesis, glycolysis and angiogenesis (26, 35-37). In addition to the genes required for metabolic adaptation to hypoxia, HIF-1α also enhances the expression of genes coding for growth factors, growth factor receptors, components of the apoptotic pathway and cell cycle regulators (26, 38, 39). Because HIF-1α and 2α can regulate the expression of genes involved not only in angiogenesis but also those that contribute to tumor cell survival and aggressiveness, these hypoxia inducible factors have been suggested as potential targets for anti-neoplastic therapy (26). Although hypoxic cells are known to be refractory to radiation and chemotherapy, the exact role of HIFs in chemo or radioresistance is yet to be established. Because the majority of solid tumors contain regions of hypoxia, hypoxia promotes angiogenesis and NSAIDs inhibit angiogenesis, we hypothesized that NSAIDs inhibit the HIF transcription factors.

We studied the effect of ibuprofen on HIF-inducible transcription factors HIF-1α and HIF-2α in PC3 and DU-145 cells. Ibuprofen treatment rapidly reduced HIF proteins under normoxic condition (Fig. 7).

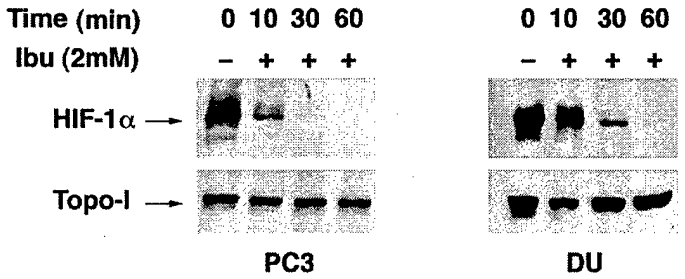


Fig. 7: PC3 and DU-145 cells were treated with different concentrations of ibuprofen under normoxia for 1h. HIF-1a protein was analyzed by western blot analysis. Topo-1 was used as loading control.

Treatment of cells with ibuprofen prior to exposure to hypoxia resulted in significant inhibition of HIF-1α at the end of 1h hypoxic gassing period (Fig. 8). At 3-4 h of hypoxia, the inhibition still persisted but was ~ 50% of the accumulation seen in cells exposed to hypoxia without ibuprofen (Fig. 9A). Ibuprofen also inhibited HIF-2α under normoxic and hypoxic conditions in both cell lines. Inhibition in HIF-2α was persistent up to 24h (Fig. 9B).

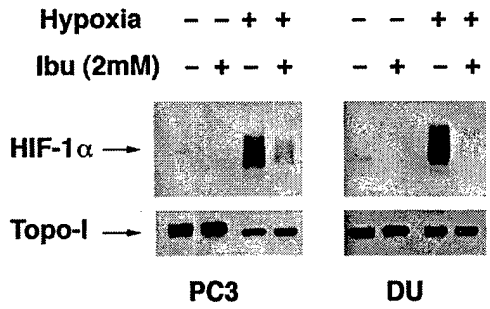


Fig. 8: Ibuprofen inhibited hypoxic accumulation of HIF-1α at 1h. PC3 and DU-145 cells were treated with 2mM ibuprofen for 30min and exposed to hypoxia for 1h. HIF-1α levels were analyzed by western analysis. Membranes were reprobed for Topo-1 as a loading control.

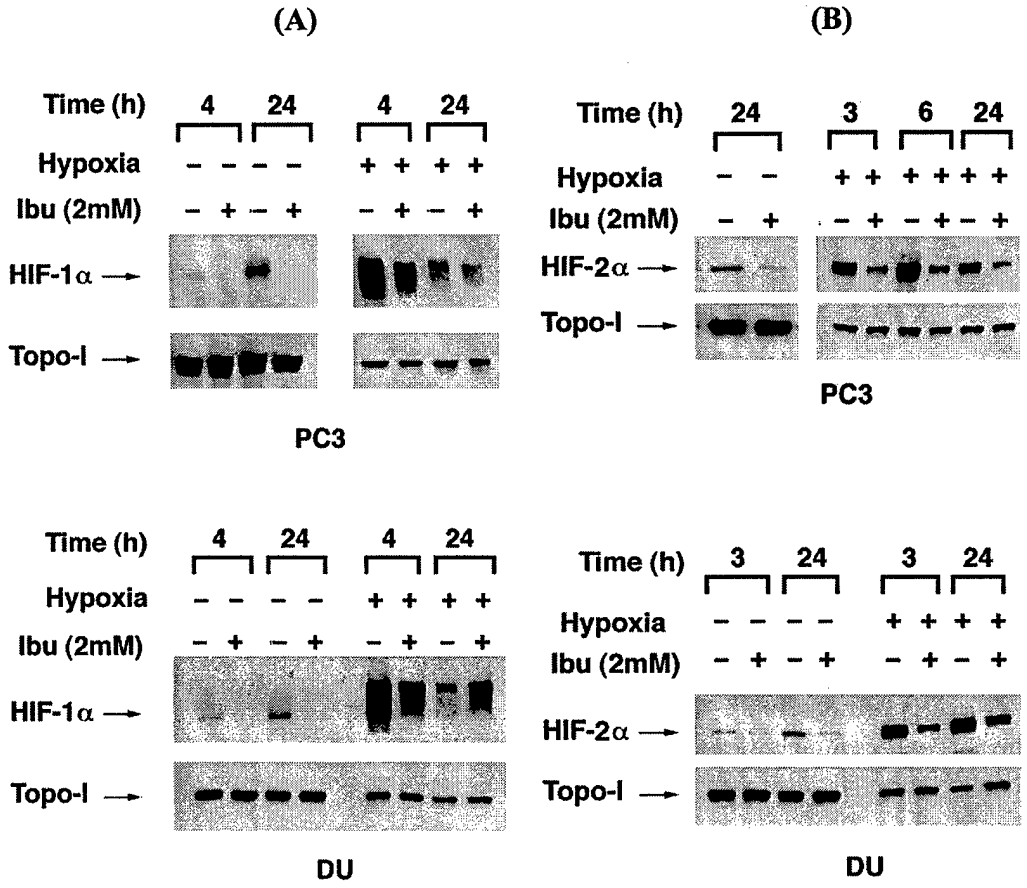


Fig. 9: Effect of ibuprofen on HIF-1α (A, left) and HIF-2α (B, right) under normoxic and hypoxic conditions at later time points. PC3 and DU-145 cells were treated with ibuprofen for 30min and exposed to hypoxia. HIFs were analyzed at the indicated times. Sixty μg protein for normoxic samples and 30μg protein for hypoxic samples were separated on 6% gel. Membranes were reprobed for Topo-1 as a loading control.

To gain insight into the potential physiological significance of the inhibitory effect of ibuprofen on HIFs, the expression of two HIF-regulated gene products-VEGF and Glut-1, was also determined (VEGF). Reduction in HIF levels resulted in the subsequent reduction in the

HIF-regulated gene products VEGF (Fig. 10A) and Glut-1 in hypoxic cells (Fig. 10B). Reduction in VEGF and Glut-1 was seen only at a concentration at which HIF-1α was inhibited. These data suggest that the anti-angiogenic actions of NSAIDs may be mediated at least in part via a decrease in these hypoxia-inducible factors.

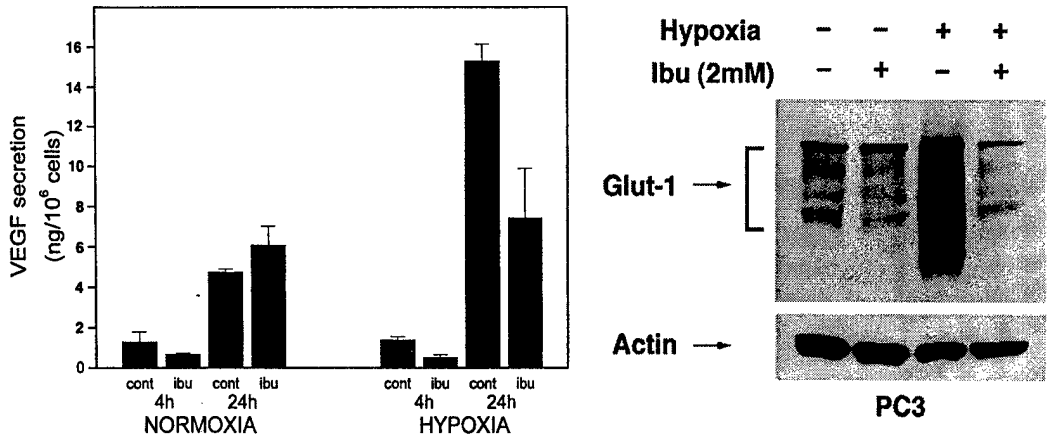


Fig. 10: Ibuprofen inhibited VEGF (left) and Glut-1 (B) under hypoxic condition in PC3 cells. VEGF protein secreted in media was analyzed by ELISA assay and Glut-1 protein in cell extracts was analyzed by western blot analysis at 24h after treatment. Similar effects were seen in DU-145 cells. After probing for Glut-1, the membrane was reprobed for actin as a loading control.

To determine whether the inhibition of HIF-1α was specific to ibuprofen or it was a feature shared by other NSAIDs also, PC3 and DU-145 cells were treated with non specific NSAIDs diclofenac, ketorolac and indomethacin. At the concentrations studied, these NSAIDs also inhibited HIF-1α (Appendix, ***Figure*** 4A) under normoxia and hypoxia (Appendix, ***Figure*** 4B), to varying degrees.

Treatment of PC3 and DU-145 cells with COX-2 specific NSAID NS398 at 100μM concentration under the same experimental conditions (10% serum media) failed to inhibit HIF-1α (**Fig. 11A**). When cell were treated with NS398 in 0.1% serum media slight inhibition of HIF-1α was observed (data not shown), so we treated PC3 cells in 0.1% serum media with 100μM NS398 for 1h and then exposed to hypoxia. There was essentially no effect on hypoxic accumulation of HIF-1α with NS398 at 1h (Fig. 11B) as compared to the significant inhibition seen with 2mM Ibu (Fig. 8) at this time point. Furthermore, treatment with 100μM NS398 failed to inhibit the HIF-regulated proteins VEGF (Fig. 11C) and Glut-1 (Fig. 11D) at 24h.

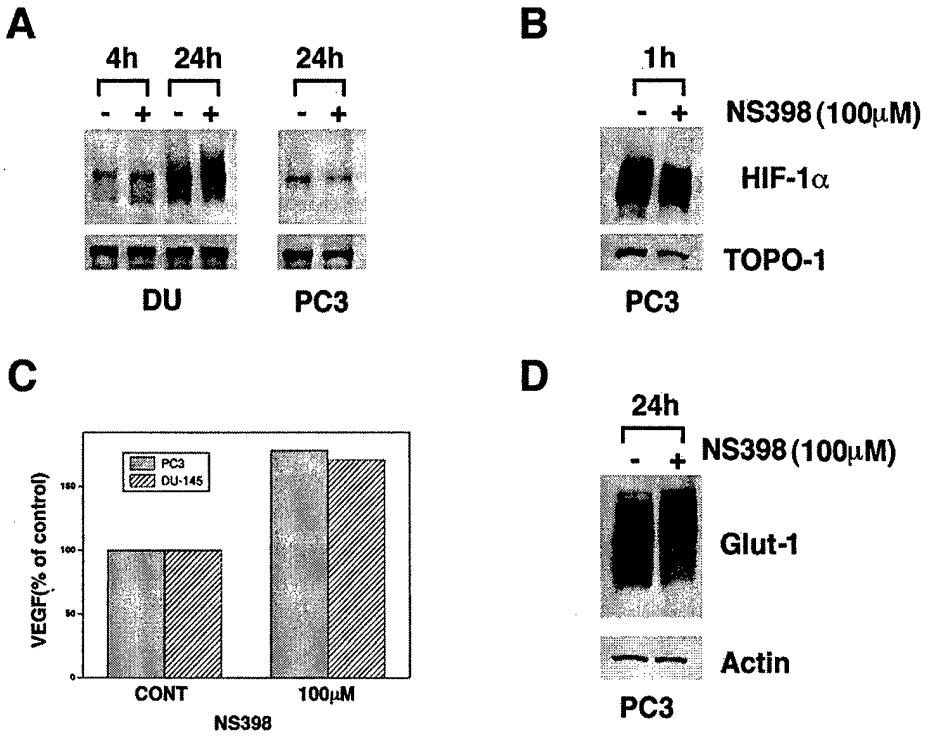


Fig. 11: (A) Effect of COX-2 specific NSAID NS398 on HIF-1α under normoxic condition. DU-145 and PC3 cells were treated with 100μM NS398 in 10% serum media for the times indicated. (B) PC3 cells were treated with 100μM NS398 in 0.1% serum media for 1h and then exposed to hypoxia. HIF-1α levels were analyzed at 1h. (C) VEGF levels (% of control) at 24h in PC3 and DU-145 cells treated with 100μM NS398 and exposed to hypoxia. This experiment was performed twice and data from a representative experiment is shown. (D) Glut-1 levels at 24h in PC3 cells treated with 100μM NS398 in 0.1% serum and exposed to hypoxia. Membranes were reprobed for TOPO-1 or actin as loading controls.

The anti-angiogenic effect of NSAIDs such as ibuprofen has been suggested to involve the COX-2 (11, 12, 14-16). PC3 cells expressed similar levels of COX-2 under normoxic and hypoxic conditions. Ibuprofen treatment resulted in an increase in COX-2 levels in PC3 cells under both conditions. However, with respect to a role for COX-2 in the ibuprofen-mediated reduction in HIF-1α and HIF-2α levels, the most significant data was obtained from DU-145 cells. In this cell line COX-2 protein was not detected and no increase was detected after hypoxia or ibuprofen treatment (Fig. 2B). These data suggest that COX-2 does not mediate the effects of ibuprofen on HIF protein levels.

5. Regulation of HIF:

HIF proteins are very labile under normoxic condition and are rapidly degraded. The protein product of von Hippel-Lindau (VHL) gene functions as a part of a multiprotein complex that in the presence of O_2 binds to HIFs and leads to their rapid degradation by ubiquitination and degradation in 26S proteasomes (28-31). VHL disease is a hereditary cancer syndrome where affected people develop blood-vessel tumors, called hemangioblastomas. The VHL gene is a tumor suppressor gene. In VHL patients the gene is inactivated by mutation or hypermethylation in susceptible cell. Since HIFs regulate angiogenesis by inducing angiogenic factors VEGF and VEGF receptor flt/1, the inhibition of HIF degradation caused by mutations in pVHL results in uncontrolled proliferation of blood vessels (31).

Since prostate cancer cells expressed HIF-1α and HIF-2α under normoxic condition, we examined the VHL status of prostate cancer cells. Renal carcinoma cells 786-O-WT expressing wild type VHL was used as positive control. VHL protein is expressed in two forms-$pVHL_L$ (~28-30kD) and VHL_S (~17-19kD). Western blot analysis after immunoprecipitation of cell extracts with anti-VHL antibody revealed that although prostate cancer cells did not express VHL large, they did expressed VHL small. RT-PCR analysis confirmed the presence of VHL gene products in prostate cancer cell lines (Fig. 12).

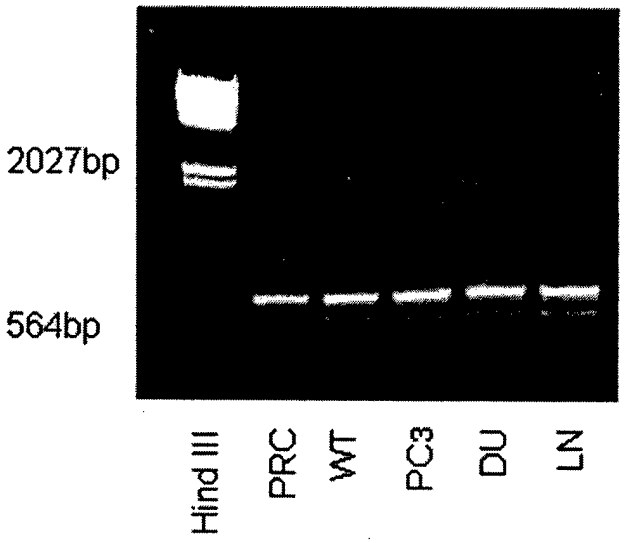
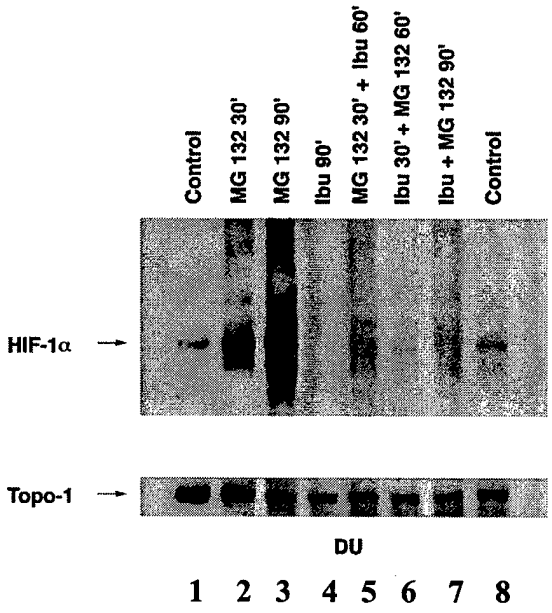


Fig. 12 : RT-PCR analysis of VHL in 786-0-renal carcinoma cells and prostate carcinoma cells. Total RNA was prepared using TRIzol reagent and 2μg RNA was used as a template for cDNA synthesis using oligo-dT primer. VHL forward primer-5' TGGTCTGGATCGCGGAGGGA 3' and VHL reverse primer-5' TCAAGACTCATCAGTACCATCAAAAG 3' were used for each PCR reaction. Thermocycling conditions were as follows: 30 cycles at 95^0C denaturation for 1min, 59.5^0C annealing for 30 s and 72^0C extention for 10 min. PCR products were separated on 1.25% agarose gel using Hind III DNA marker. The expected product length was 723bp.

Degradation of HIF by proteasomal pathway:

Under normoxic condition HIFs bind to pVHL and are rapidly ubiquitinated and degraded by proteasomal pathway. In order to determine if the inhibition of HIFs by ibuprofen under normoxic condition was mediated by proteasomal pathway, we studied the effect of proteasomal inhibitor MG132 on HIF-1α degradation in the presence and absence of ibuprofen in prostate cancer cells (Fig. 13). MG132 inhibited HIF degradation and increased HIF levels rapidly (see lane 3: -30min with MG132, lane 4: -90min with MG132). When cells were pretreated with ibuprofen and then treated with MG132 (lane 6) or incubated together with ibuprofen and MG132 (lane 7), HIF accumulation expected at 90min (lane 4) was inhibited. When ibuprofen was added after pretreating cells with MG132, HIF was still inhibited, but to a lesser extent (compare lane 4 and lane 5). These studies suggest that degradation of HIF by ibuprofen was mediated via proteasomal degradation pathway.



lane 1: DMSO control
lane 2: MG132 30min
lane 3: MG132 90min
lane 4: ibu 90min
lane 5: ibu added 30min after adding MG132
lane 6: MG132 added 30min after adding ibu
lane 7: ibu and MG132 added together for 90min
lane 8: DMSO control

Fig. 13: Effect of ibuprofen on MG132 mediated HIF-1α accumulation. DU-145 cells were treated with proteasomal inhibitor MG132 and ibuprofen as indicated. Similar effects were seen in PC3 cells. Membrane was reprobed for Topo-1 as a loading contol.

Work in progress

1) Role of HIF in radiation response

Solid tumors contain areas hypoxic regions or experience intermittent hypoxia. Recent studies have indicated that the majority of common human tumors express HIF-1α and/or HIF-2α whereas adjacent normal tissues do not (40). Although hypoxia makes tumor cells more resistant to radiation therapy the role of HIFs in radioresistance is not established. We are now comparing the radiosensitivity of cells over expressing HIF-1α or -2α with matched cell lines expressing basal levels of HIF-1α and HIF-2α.

We are also studying the effect of radiation on HIF protein levels. Preliminary data showed that radiation had no effect on HIF levels under normoxic condition in prostate cancer cells (Fig. 14).

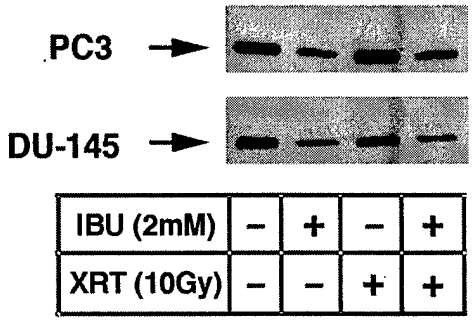


Fig. 14: Effect of radiation and ibuprofen on HIF-2α. PC3 and DU-145 cells were treated with 2mM ibuprofen and irradiated after 1h. HIF-2α levels were analyzed at 24h by western blot analysis.

2) Effect of NSAIDs on cells over expressing HIFs

Over 50% of sporadic renal carcinoma cells and clear cell renal carcinomas over express HIFs due to mutations in VHL (31). We examined the effect of ibuprofen and other NSAIDs in renal carcinoma cell lines which over expressed HIF-1α (C2 cells) or HIF-2α (786-0-PRC cells) due to lack of functional VHL and compared them with C2/VHL and 786-0-WT cells which showed basal levels of HIF-1α (C2/VHL) or HIF-2α (786-0-WT), due to forced over expression of VHL (Figs. 15, 16). Preliminary studies showed that treatment with ibuprofen and other NSAIDs for 24h upregulated VHL protein in C2/VHL (data not shown) and 786-0-VHL cells (Fig. 15). There was a corresponding reduction in basal HIF-2α in 786-0-WT cells (Fig. 15) and HIF-1α in C2/VHL cells (Fig. 16). However, the NSAIDs had no effect on the over expressed HIF-2α in 786-0-PRC cells or over expressed HIF-1α in C2 cells which lacked functional VHL (Figs. 15, 16). These studies suggest that NSAIDs may interfere with HIF/VHL pathway under normoxic condition but are unable to degrade HIFs in cells that lack functional VHL.

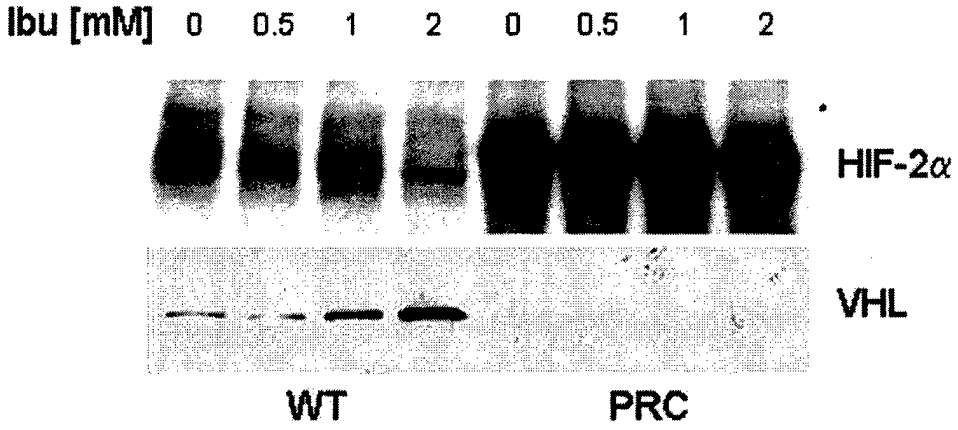


Fig. 15: Effect of ibuprofen on HIF-2α (top panel) and VHL (bottom panel) in 786-0-WT (lanes 1-4) and 786-0-PRC cells (lanes 5-8). Cells were treated with different concentrations of ibuprofen for 24h. HIF-2a was analyzed on 6% gel and VHL was analyzed on 14% gel.

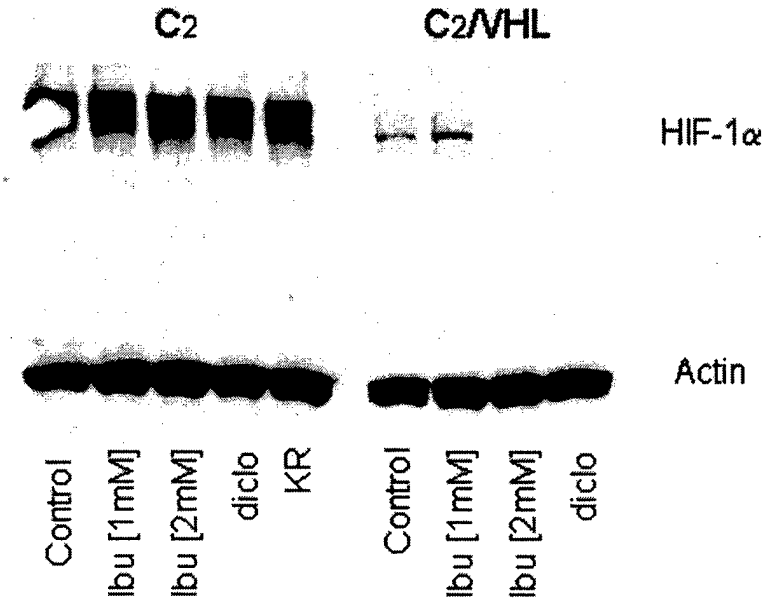


Fig. 16: Effect of nonspecific NSAIDs on HIF-1α in C2 and C2/VHL cells. Cells were treated with ibuprofen (ibu), diclofenac (diclo) and ketorolac (KR) for 24h and HIF-1α was analyzed on 8% gel. Membrane was reprobed for actin as a loading control.

3) Effect of NSAIDs on angiogenesis

Several studies have demonstrated that NSAIDs inhibit tumor growth by inhibiting angiogenesis (20, 41-43). NSAID-mediated inhibition of angiogenesis is generally attributed to the inhibition of COX-2, since NSAIDs are primarily associated with the inhibition of cyclooxygenases (17-21). It is well documented that in addition to COX-2, angiogenesis is also regulated by HIFs under hypoxia by increasing angiogenic factors including VEGF and VEGFreceptor, Flt-1 (23, 26, 35, 38). Our studies have demonstrated that ibuprofen inhibited VEGF secretion by tumor cells *in vitro* under hypoxic environment. However, in vivo, NSAIDs are bound have an effect on tumor stromal cells and endothelial cells.

The role of NSAIDs in the inhibition of angiogenesis will be determined by studying the effect of NSAIDs on endothelial proliferation and migration. Endothelial tube formation will be studied on matrigel. In addition we will treat co-cultures of endothelial cells and tumor cell with NSAIDs alone or in combination with radiation. We will also study the effect of NSAIDs on endothelial cells under hypoxia. In these studies either each component or both components will be treated. A broad concentration range of NSAIDs will be used to separate the COX-2-dependent effects (lower concentrations) and COX-2-independent effects (HIFs?) of NSAIDs on angiogenesis.

4) NSAIDs and PPAR activation

Peroxisome proliferator-activated receptors belonging to PPAR family of transcription factors that regulate lipid metabolism have been recently shown to be involved in tumor progression (41-48). The ligands for PPAR include synthetic antidiabetic thiazolidinediones and cyclopentane prostaglandins, of which 15d-PGJ_2 are the most potent (43). Activation of PPAR-γ results in the inhibition of the expression of genes involved in angiogenesis, which include VEGF receptors Flk/KDR, Flt-1 and uPA (42). PPAR agonists also induce COX-2 (7). PPAR-γ agonists regulate cytokine gene expression as they inhibit transcription factors NF-kB, AP-1 and STAT (49) and suppress production of monocyte inflammatory cytokines (50). PPAR agonists inhibit cell growth and have been shown to induce apoptosis in endothelial cells (51) as well as cancer cells (45, 47). It has been shown that human prostate cancer cell lines, PC3, DU-145, and LNCaP, express PPAR-γ and treatment of cells with 15d-PGJ_2 induced cell death (47, 52). PPAR activators have been shown to be effective *in vivo* and *in vitro* for prostate cancer cells (44, 47, 52). Moreover, PPAR-γ ligand Δ^{12}-prostaglandin J2 exhibited synergistic cytotoxicity with ionizing radiation in 289 melanoma and LNCaP prostate cancer cells (53).

Interestingly, NSAIDs also activate PPAR-γ and disrupt the ability of PPAR-δ to bind to its recognition sequence (54). Regulation of PPAR transcription factors may be one of the important mechanisms for cancer prevention, therapy and/or radiosensitization by NSAIDs. In order to investigate if PPAR γ is a target for radiosensitization by NSAIDs, the effect of PPAR ligands on clonogenic survival of irradiated PC3 and DU-145 cells is currently under investigation.

The effect of radiation and ibuprofen on PPAR-γ was examined by western blot analysis in prostate cancer cells (Fig. 17). Preliminary data showed that in PC3 cells the lower band,

PPAR-γ 2, was reduced at 24h after 8Gy irradiation. Ibuprofen reduced the upper slow migrating phosphorylated form of PPAR γ in a dose-dependent fashion (data not shown) with a significant reduction at 2 mM concentration (Fig. 17). In the presence of ibuprofen the radiation-induced inhibition of PPAR-γ2 was abolished and PPAR profile was similar to that seen in cells treated with ibuprofen alone. The effects of ibuprofen and radiation on PPARs in DU-145 cells were different than the PC3 cells. In DU cells there was an overall increase in both forms of PPARs.

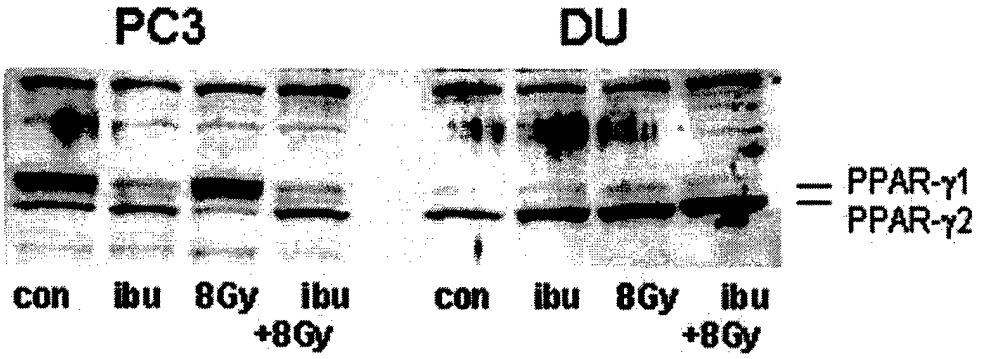


Fig. 17: PC3 and DU-145 cell were treated with 2mM ibuprofen and exposed to 8Gy after 4h. Cell extracts were prepared after 24h and analyzed for PPAR-γ by western blot analysis.

5) Effect of NSAIDs on molecular targets in vivo

We plan to study the effect of NSAIDs on the various molecular targets in tumors *in vivo*. In preliminary studies we were able to detect HIF-1α in DU-145 tumors grown in nude mice by western blot analysis (Fig. 18). HIF-1α was not expressed in kidney or liver. We plan to look for other NSAID targets identified in the present study including COX, HIFs, VEGF and Glut-1 by western blot analysis and by immunohistochemistry and evaluate the effects by treating animals with NSAIDs alone or in combination with radiation. Other *in vivo* angiogenesis markers such as factor VIII, CD31 and microvessel density will be also examined.

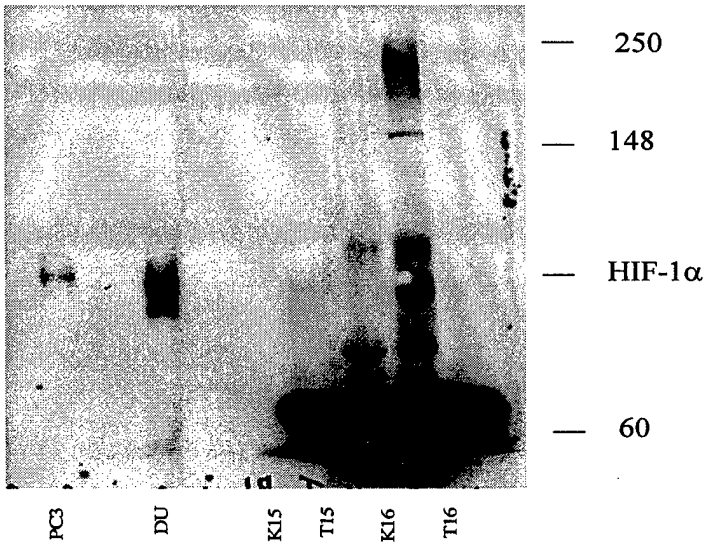


Fig. 18: HIF-1α expression in DU-145 tumor and kidney. DU-145 tumors were grown in nude mice. Animals were sacrificed and tumors (T15, T16) and kidneys (K15, K16) were removed. Tissues were homogenized and extracts were separated on a 6% gel and probed with HIF-1α antibody. PC3 and DU: extracts of cells grown in tissue culture.

Research accomplishments:

We have identified novel cellular targets for NSAIDs focusing primarily on ibuprofen. These include 1) COX-2 protein, 2) NFkB and the IKK-kinase that regulates NFkB degradation, 3) Hypoxia-inducible transcription factors HIF-1α and HIF-2α and the gene products (VEGF and Glut-1) regulated by HIFs, and 4) VHL protein that regulates normoxic degradation of HIFs

Our results are the first demonstration that NFkB and IKK-α is constitutively activated in hormone-independent PC3 and DU-145 prostate carcinoma cells but not in hormone-sensitive LNCaP cells.

Although HIFs are known to be very labile under normoxic condition, we have shown that HIFs were expressed in prostate cancer cell lines under normoxic condition. We showed that HIF-1α disappeared rapidly when conditioned media was removed suggesting a role for growth factors in normoxic HIF expression.

We have shown that ibuprofen rapidly inhibited HIFs under normoxic condition, reduced initial hypoxic accumulation of HIFs and subsequently inhibited HIF-regulated gene products VEGF and Glut-1 under hypoxic condition. *In vitro* these effects were seen at concentrations higher than those required to inhibit prostaglandin synthesis suggesting a COX-2 independent mechanism.

Ibuprofen inhibited HIF-1α accumulation in cells treated with proteasomal inhibitor MG132, indicating a role for ibuprofen in proteasomal degradation pathway.

Using cells over expressing VHL we have shown that ibuprofen up regulates VHL protein. Using counter parts of these cells, which lack functional VHL and therefore over express HIF-1α or HIF-2α, we have shown that ibuprofen mediates degradation of HIFs by VHL pathway. HIFs were not degraded in cells that lacked functional VHL.

Reportable Outcomes:

Publications:

S. T. Palayoor, M. Y. Youmell, S. K. Calderwood, C. N. Coleman and B. D. Price, Constitutive activation of IKB-kinase a and NFkB in prostate cancer cells is inhibited by ibuprofen. Oncogene, 18, 7389-7394, 1999.

S. T. Palayoor, P. J. Tofilon and C. Norman Coleman, Inhibition of HIF-1a and HIF-2a by ibuprofen in prostate cancer cells, in revision, Cancer Research, 2002.

Abstracts:

S. T. Palayoor, S. K. Calderwood, E. A. Bump and C. Norman Coleman, Role of eicosanoid inhibitors in radiosensitization of PC3 prostate carcinoma cells. 47th Radiation Research society Meeting, 2000, Albuquerque, NM.

S. T. Palayoor and C. Norman Coleman, Inhibition of HIF-1a and VEGF by ibuprofen in PC3 human prostate cancer cells. 48th Radiation Research Society Meeting, 2001, San Juan, Puerto Rico.

S. T. Palayoor, P. J. Tofilon and C. Norman Coleman, Inhibition of HIF-1α by ibuprofen under normoxia and hypoxia. 49th Radiation Research society Meeting, 2002, Reno, Nevada.

Conclusions:
Future direction

During the tenure of this grant we looked for potential molecular targets of NSAIDs, which may be involved in the observed radiosensitization of prostate cancer cells. The studies reported here were mostly conducted *in vitro* and it became apparent that although NSAIDs inhibited prostaglandin synthesis at micromolar concentrations, several other effects of NSAIDs were seen at concentrations that were well above those that inhibit of prostaglandin synthesis (1, 5-10, 14, 22, 55). These included induction of apoptosis, cytotoxicity, radiosensitization, and inhibition of various transcription factors along with the inhibition of target proteins regulated by these transcription factors. The plasma concentrations of NSAIDs that can be achieved are at least 5-10fold lower that the concentrations that are effective *in vitro*. Even if these concentrations can be achieved *in vivo*, at these concentrations nonspecific NSAIDs have adverse toxicity problems. *In vitro*, even the COX-2 specific inhibitors appear to have a prostaglandin-dependent and prostaglandin-independent component (22). The COX-2 specific NSAIDs which are less toxic did not appear to be effective radiosensitizers *in vitro* or required much higher concentrations than those that are required to inhibit prostaglandin synthesis (56). Nevertheless, NSAIDs appear to be quite effective in inhibiting tumor growth either alone or in combination with radiation in animal tumor models at the plasma concentrations that can be achieved *in vivo*. The significance of inhibition of HIFs and HIF-regulated gene products by NSAIDs clearly needs to be further investigated in *in vivo* models.
The *in vivo* inhibition of angiogenesis and tumor growth by some of the COX-2 specific NSAIDs is recently attributed to the NSAID-mediated inhibition of host stromal and angiogenic vasculature COX-2 (20, 21). Therefore it is necessary to determine the effect of NSAIDs on angiogenesis using complex *in vitro* models and *in vivo* tumors.

We have initiated DNA array analysis to find genes modulated by low concentrations of NSAIDs versus high concentrations of NSAIDs.

The specific role of COX-2 and HIFs will be studied using siRNA technique.

Future experiments:

1) Study radiosensitizing potential of a panel of COX-2 specific NSAIDs *in vivo* since they are less toxic than non-specific NSAIDs.
2) We are studying the effect of a combination of COX inhibitors, ibuprofen plus COX-2 inhibitors in that they may produce synergistic effects. This would provide a more useful clinical strategy than using a single inhibitor.
3) The role of NSAIDs in the inhibition of angiogenesis will be determined by studying the effect of NSAIDs on endothelial proliferation, migration and tube formation on matrigel. In addition we will treat co-cultures of endothelial cells and tumor cell with NSAIDs alone or in combination with radiation. In this study either each component or both components will be treated. A broad concentration range of NSAIDs will be used to separate the COX-2-dependent effects (lower concentrations) and COX-2-independent effects.
4) We plan to study the effect of NSAIDs on the various molecular targets in tumors *in vivo*. In preliminary studies we detected HIF-1α in tumors grown in nude mice by western blot analysis.

HIF-1α was not detected in kidney or liver. We plan to look for other NSAID targets identified in the present study including COX, HIFs, VEGF and Glut-1 by western blot analysis and by immunohistochemistry and evaluate the effect of NSAIDs in combination with radiation.
5) We remain interested in a clinical trial of NSAIDs with radiation. We would do so in the circumstance that a combination of NSAIDs (Future experiments #2) produces inhibition of the molecular targets at clinically achievable concentrations. Any study would require assessing molecular effects of the inhibitors including assessing prostaglandin profiles, COX activity and impact on non-COX targets including NFkB, HIF and PPARs.
6) In that the field has evolved rapidly since this application, our work has opened up two very fruitful new molecular targets for modifying the radiation response, HIF and PPARs, in addition to the NFkB. We will expand our work to brain tumors.

"So What" Section.
First of all, we are grateful for the support from the DOD. What we have done is help put some science and logic behind the COX studies as anticancer agents. There is now a wide array of relatively empiric clinical studies being conducted. By empiric, we mean that there are few if any biological correlates to understand how the drug is working and whether it is impacting specific targets. The drug concentrations achievable in the clinic will inhibit the COX enzymes but it is not known whether or not other non-COX targets are relevant.

Our studies have defined a set of non-COX targets that may be relevant and that should be assessed in clinical trials. These include the NFkB pathway, the HIF pathway and downstream effectors such as VEGF and possibly the PPAR pathways. The need to use multiple agents to disrupt a target is a lesson learned repetitively in oncology and infectious diseases. Thus, our ongoing approach to using multiple COX inhibitors and/or a COX inhibitor plus another agent targeting the pathway may prove to be a useful clinical strategy. In that regard, our work has opened up two very interesting pathways for radiation modification, the HIF and PPAR pathways.

The support provided by this grant has also been instrumental in recruiting faculty for the program to which the PI moved, to the National Cancer Institute, Radiation Oncology Branch. The molecular targeted approach used in this research will be complemented by novel imaging and normal tissue studies, allowing us to "credential" molecular targeted therapy for radiation oncology.

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Appendices

1) Fig.1: NSAIDs inhibit prostaglandin synthesis at micromolar concentrations.
2) Fig.2: Effect of NSAIDs, COX-2 inhibitors and 5-lipoxygenase inhibitors on COX-2 protein in PC3 cells.
3) Table I: Effect of recombinant human IL-6 (rhIL-6) on clonogenic survival of PC3 cells treated with ibuprofen and radiation.
4) Fig.3: TNF-α secretion by PC3 cells treated with radiation and ibuprofen.
5) Fig.4: Effect of nonspecific NSAIDs on HIF-1α under normoxic and hypoxic condition.
6) Abstract: S. T. Palayoor, S. K. Calderwood, E. A. Bump and C. Norman Coleman, Role of eicosanoid inhibitors in radiosensitization of PC3 prostate carcinoma cells. 47th Radiation Research society Meeting, 2000, Albuquerque, NM.
7) Abstract: S. T. Palayoor and C. Norman Coleman, Inhibition of HIF-1a and VEGF by ibuprofen in PC3 human prostate cancer cells. 48th Radiation Research Society Meeting, 2001, San Juan, Puerto Rico.
7) Abstract: S. T. Palayoor, P. J. Tofilon and C. Norman Coleman, Inhibition of HIF-1α by ibuprofen under normoxia and hypoxia. 49th Radiation Research society Meeting, 2002, Reno, Nevada.

Publications:

S. T. Palayoor, M. Y. Youmell, S. K. Calderwood, C. N. Coleman and B. D. Price, Constitutive activation of IKB-kinase a and NFkB in prostate cancer cells is inhibited by ibuprofen. Oncogene, 18, 7389-7394, 1999.

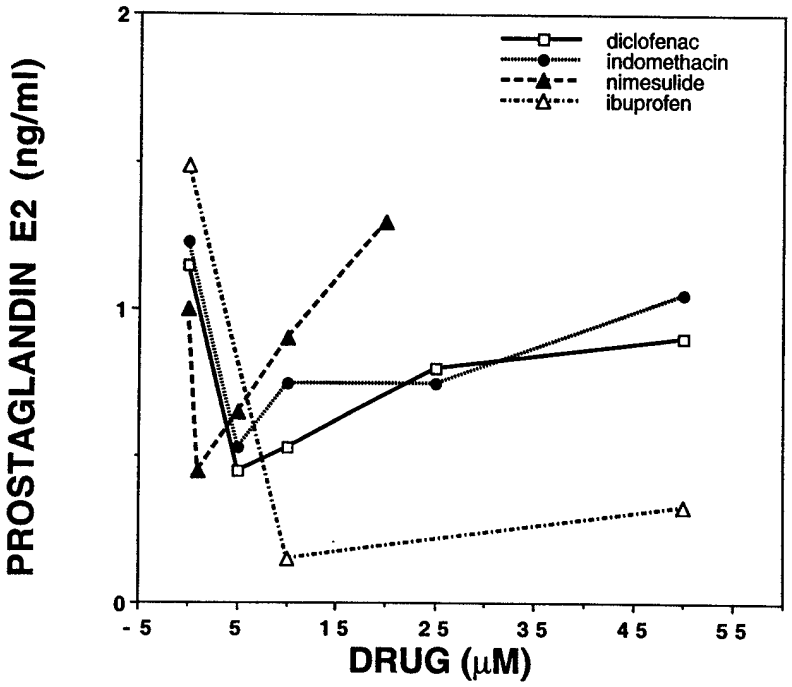
S. T. Palayoor, P. J. Tofilon and C. Norman Coleman, Inhibition of HIF-1α and HIF-2α by ibuprofen in prostate cancer cells, *in revision*, Cancer Research, 2002.

List of personnel receiving pay from the research effort: None

Appendix

Figure 1

NSAIDs inhibit prostaglandin synthesis at micromolar concentrations

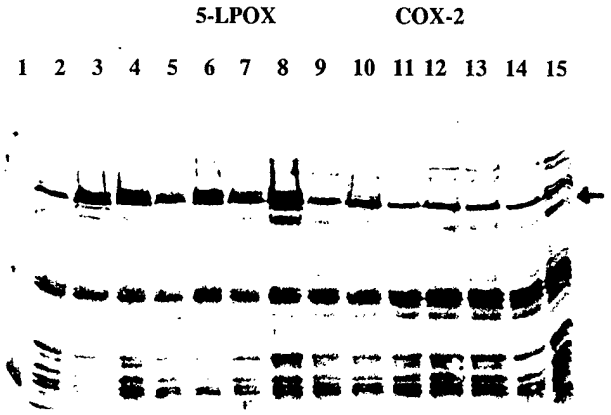


The effect of ibuprofen and other NSAIDs on arachidonic acid (AA)-induced prostaglandin E2 (PGE2) synthesis was determined by ELISA assay (Oxford Biomedical Res, Inc.). PC3 cells were plated in 6-well plates and treated for 10min with NSAIDs in the presence of 30μM AA. Media was collected and analyzed for PGE2. The basal PGE2 levels were 0.36 ± 0.07 ng/ml (n=8). AA increased PGE2 to 1.33 ± 0.18 ng/ml. NSAIDs inhibited PGE2 synthesis at micromolar concentrations. Nimesulide is a COX-2 specific inhibitor.

Appendix

Figure 2

Analysis of COX-2 protein in PC3 cells treated with eicosanoid inhibitors



lanes 1: mol.wt.marker
lane 2: control
lane 3: 1 mM ibu
lane 4: 2 mM ibu
lane 5: 10 μM MK 886
lane 6: 50 μM MK 886
lane 7: 10 μM Rev 5901
lane 8: 50 μM Rev 5901
lane 9: 10 μM NS 398
lane 10: 50 μM NS 398
lane 11: 10 μM nimesulide
lane 12: 50 μM nimesulide
lane 13: 10 μM niflumic acid
lane 14: 50 μM niflumic acid
lane 15: DMSO control

Cells were treated with nonspecific NSAID ibuprofen (lanes 3, 4), 5-lipoxygenase inhibitors (lanes 5-8) and COX-2 specific inhibitors (lanes 9-14) for 24h. COX-2 protein was analyzed by western blot analysis using alkaline phosphatase-conjugated secondary antibody for detection. Although COX-2 inhibitors did not show increase in COX-2 protein at the concentrations used here, our recent data showed an increase in COX-2 protein when cells are treated with 100μM NS 398.

Appendix

TABLE 1

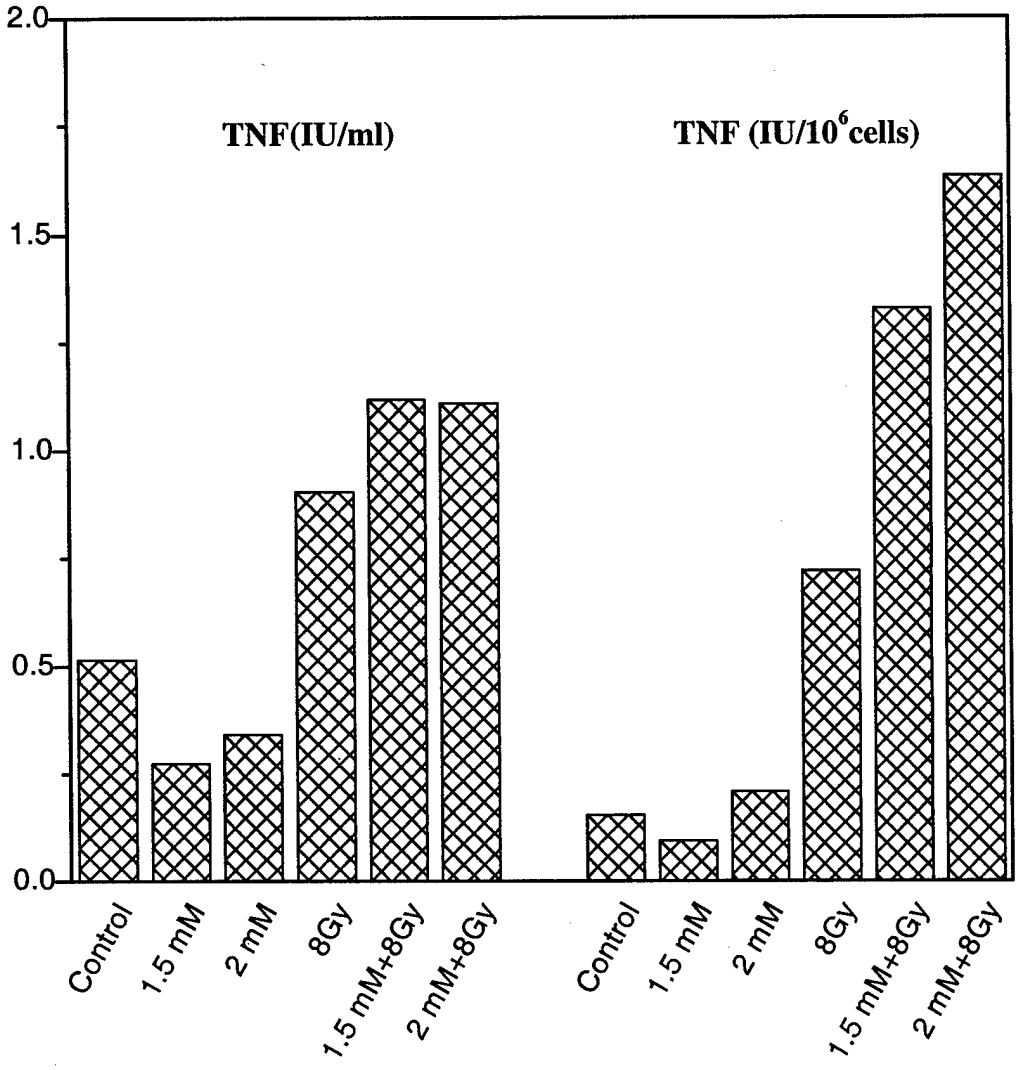
Recombinant IL-6 partially reversed radiosensitization of PC3 cells by ibuprofen

Treatment	PE expt /PE control Avg ± SD (n=2)	Divided by IL-6 survival	Divided by Ibu survival	IL6 protection factor
Control	1.00			
4 Gy	0.32 ± 0.06			
1.5 mM ibu	0.56 ± 0.32			
1.5 mM ibu + 4 Gy	0.06 ± 0.05		0.11	
10ng/ml rhIL-6	0.87 ± 0.07			
rhIL-6 + 4 Gy	0.27 ± 0.02	0.31 ± 0.02		1.00 ± 0.16
rhIL-6 + ibu	0.55 ± 0.24	0.63 ± 0.24		1.26 ± 0.16
rhIL-6 + ibu + 4 Gy	0.08 ± 0.06	0.10 ± 0.08	0.15	1.48 ± 0.05

PC3 cells were treated with ibuprofen in high glucose low pH buffer. After 1 h cells were irradiated with 4 Gy. Ibuprofen was removed after another 1 h and cells were then incubated in serum-free and drug-free media. After 24 h cells were plated for clonogenic survival assay in complete media. rhIL-6 was added during ibuprofen treatment and also during the 24 h incubation period before plating cells fo clonogenic asay. The data suggested that rhIL-6 partially reversed radiosensitization of PC3 cells by ibuprofen.

Appendix
Figure 3

Effect of ibuprofen on TNF-α levels in conditioned media from PC3 cells



PC3 cells were exposed to 8Gy gamma irradiation in the presence or abscence of ibuprofen. TNF-α levels in the media collected on day 5 were measured by ELISA assay (R and D systems).TNF-α was increased in media collected from cells exposed to 8 Gy. Ibuprofen did not inhibit the radiation-induced increase in TNF-α.

Appendix

Figure 4

Effect of nonspecific NSAIDs on HIF-1α levels in PC3 and DU-145 cells under normoxic and hypoxic conditions

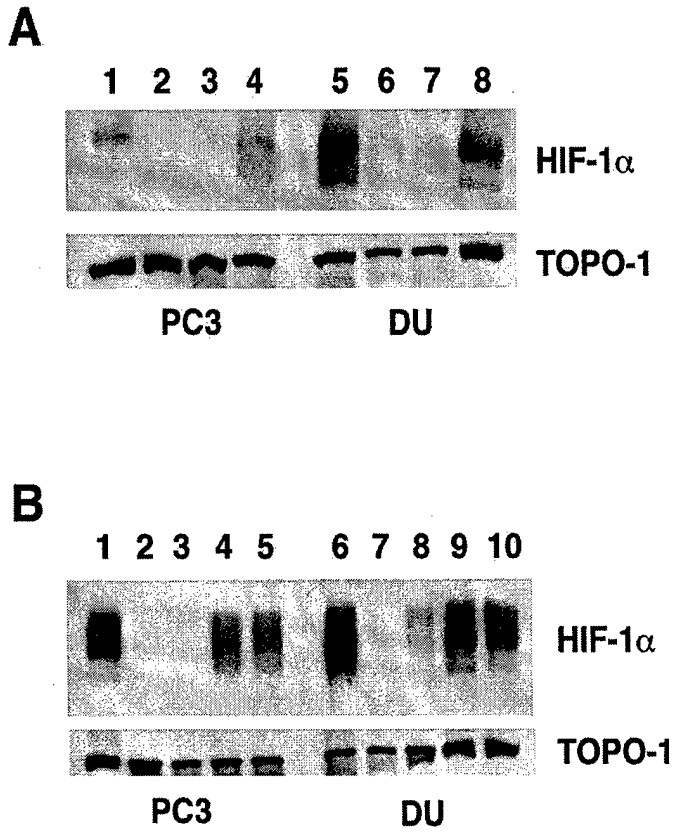


Figure 4: Effect of nonspecific NSAIDs on HIF-1α levels in PC3 and DU-145 cells under A) normoxic and B) hypoxic condition. (A) HIF-1α levels in cells treated with NSAIDs for 1h. Control: (lanes 1, 5), 2mM Ibu: (lanes 2, 6), 0.3mM diclofenac: (lanes 3, 7) and 2mM ketorolac: (lanes 4, 8). (B) HIF-1α levels in cells treated with NSAIDs for 1h followed by 1h of hypoxia. Hypoxia alone: (lanes 1, 6), 2mM Ibu: (lanes 2, 7), 0.3mM diclofenac: (lanes 3, 8), 2mM ketorolac: (lanes 4, 9) and indomethacin: (lanes 5, 10). HIF-1α levels were determined by western analysis.

Appendix

ABSTRACT

Role of eicosanoid inhibitors in radiosensitization of PC3 human prostate carcinoma cells. S. T. Palayoor[1], S. K. Calderwood[2], E. A. Bump[3] and C. Norman Coleman[1]. [1] National Institutes of Health, Bethesda, MD, 20892, [2]Dana Farber Cancer Institute, Boston, MA, 02115 and [3]Cleveland Clinic Foundation, Cleveland, Ohio, 44195. Presented at 48th Radiation Research Society Meeting, 2001, San Juan, Puerto Rico.

Our previous studies have shown that ibuprofen, a nonsteroidal antiinflammatory agent (NSAID), enhances the effects of radiation on prostate cancer cells *in vitro* as well as *in vivo*. To determine if COX-2 was the target for the NSAID toxicity we studied the effect of ibuprofen and other NSAIDs on arachidonic acid (AA)-induced prostaglandin synthesis by ELISA and found that NSAIDs inhibited the prostaglandin synthesis at much lower concentrations than those required to induce cytotoxicity and radiosensitization. We studied the effect of higher concentrations of NSAIDs on COX-2 protein by western blot analysis. PC3 cells were found to express COX-2 constitutively. Treatment of PC3 cells with higher concentration of NSAIDs further increased COX-2 protein levels at 24h. No change in COX-2 level was observed in irradiated cells. COX-2 specific inhibitors NS398, Nimesulide and Niflumic acid were less cytotoxic and showed only slight effect on the clonogenic survival at 4 Gy. They had no effect on the constitutive COX-2 protein level. At higher concentrations NSAIDs can also inhibit lipoxygenase pathway of AA metabolism. 5-HETE, a product of AA by 5-lipoxygenase (5-LO) pathway is growth-stimulatory to PC3 cells. We studied the effect of two lipoxygenase inhibitors MK 886 and Rev 5901 on PC3 cells. Both agents were cytotoxic at micromolar concentrations and appear to enhance the effect of radiation, increasing cellular detachment, apoptosis and reducing the clonogenic cell survival. At cytotoxic concentrations they also increased COX-2 protein level. These studies suggest that lipoxygenase pathway may be a better target for the treatment of prostate cancer than the cyclooxygenase pathway. 5-LO inhibitors are clinically used in treatment of asthma, inflammation and hypersensitivity and appear to be well tolerated.

Supported by DOD Prostate Cancer Grant DAMD17-98-1-8605.

Appendix

ABSTRACT

Inhibition of HIF-1α and VEGF by ibuprofen in PC3 human prostate cancer cells. S. T. Palayoor and C. Norman Coleman. National Institutes of Health, Bethesda, MD, 20892. Presented at 47th Radiation Research society Meeting, 2000, Albuquerque, NM.

Vascular endothelial cell growth factor (VEGF) is a potent mitogen for tumor angiogenesis. VEGF expression is primarily regulated by hypoxia-inducible transcription factor HIF-1 α under hypoxic condition. Recent studies from many laboratories suggest that COX-2 also plays an important role in tumor angiogenesis and COX-2 inhibitors appear to be promising anti-tumor agents in *in vivo* models. We are currently evaluating the effects of NSAIDs on HIF-1 α and COX-2 and the subsequent VEGF secretion, under normoxic and hypoxic conditions. PC3 cells expressed HIF-1 α costitutively under normoxic condition. HIF-1 α levels increased following hypoxia. Ibuprofen inhibited HIF-1 α protein under normoxic condition but was less effective under hypoxic condition. COX-2 was also constitutively expressed in PC3 cells. However, the COX-2 protein was upregulated by ibuprofen under normoxic as well as hypoxic conditions. Similar results were obtained when PC3 cells were treated with NS 398, a COX-2 specific NSAID. VEGF protein level increased approximately 2.5 folds in the media collected from hypoxic cells as compared to normoxic cells. Treatment with ibuprofen inhibited the amount of VEGF secreted in media under normoxic and hypoxic conditions. The inhibition persisted even up to 24h, only in hypoxic cells.

Supported by DOD Prostate Cancer Grant DAMD 17-98-1-8605

Appendix

ABSTRACT

Inhibition of HIF-1α by ibuprofen under normoxia and hypoxia. S. T. Palayoor, P. J. Tofilon and C. Norman Coleman. Radiation Oncology Branch and the Molecular Radiation Therapeutics Branch, NCI, National Institutes of Health, Bethesda, MD, 20892. Presented at 49th Radiation Research society Meeting,2002, Reno, Nevada.

PC3 and DU-145 human prostate cancer cells express HIF-1α (hypoxia-inducible factor) protein under normoxic condition. Cell density, growth factors, pH etc. are known to modulate HIF-1α protein levels under tissue culture condition. We observed that HIF-1α protein levels decreased by changing media and then increased over 24h. Ibuprofen inhibited the constitutive HIF-1α protein as well as the protein that accumulated following media change. A time course of inhibition of HIF-1α by ibuprofen showed that at 2mM concentration HIF-1α disappeared from cells within 30 min. Although ibuprofen inhibited two HIF-1α targeted proteins, VEGF and GLUT-1, which were upregulated by hypoxia, inhibition of HIF-1α under normoxic condition did not result in the inhibition of basal VEGF in tissue culture media and inhibition of basal GLUT-1 protein in cell extracts. Under normoxic condition HIF-1α is rapidly degraded by ubiquitinylation and proteasomal degradation. Proteasomal inhibitor MG132 inhibited HIF-1α degradation and increased accumulation of HIF-1α. Preincubation or simultaneous incubation with ibuprofen inhibited the increase in HIF-1α upregulation by MG132. When cells were pretreated with MG132 and ibuprofen was added, HIF-1α was still inhibited but to a lower extent. These studies suggest that ibuprofen affects HIF-1α degradation and stabilization.

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http://www.stockton-press.co.uk/onc

SHORT REPORT

Constitutive activation of IκB kinase α and NF-κB in prostate cancer cells is inhibited by ibuprofen

ST Palayoor[2], MY Youmell[1], SK Calderwood[1], CN Coleman[2] and BD Price*,[1]

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Apoptotic pathways controlled by the Rel/NF-κB family of transcription factors may regulate the response of cells to DNA damage. Here, we have examined the NF-κB status of several prostate tumor cell lines. In the androgen-independent prostate tumor cells PC-3 and DU-145, the DNA-binding activity of NF-κB was constitutively activated and IκB-α levels were decreased. In contrast, the androgen-sensitive prostate tumor cell line LNCaP had low levels of NF-κB which were upregulated following exposure to cytokines or DNA damage. The activity of the IκB-α kinase, IKKα, which mediates NF-κB activation, was also measured. In PC-3 cells, IKKα activity was constitutively active, whereas LNCaP cells had minimal IKKα activity that was activated by cytokines. The anti-inflammatory agent ibuprofen inhibited the constitutive activation of NF-κB and IKKα in PC-3 and DU-145 cells, and blocked stimulated activation of NF-κB in LNCaP cells. However, ibuprofen did not directly inhibit IκB-α kinase. The results demonstrate that NF-κB is constitutively activated in the hormone-insensitive prostate tumor cell lines PC-3 and DU-145, but not in the hormone responsive LNCaP cell line. The constitutive activation of NF-κB in prostate tumor cells may increase expression of anti-apoptotic proteins, thereby decreasing the effectiveness of anti-tumor therapy and contributing to the development of the malignant phenotype.

Keywords: NF-κB; ibuprofen; prostate; tumor; IKKα; androgen

The Rel/NF-κB family of transcription factors are activated by a wide range of stimuli, including DNA damage, cytokines and free radicals (Baeuerle and Baltimore, 1996; Miyamato and Verma, 1995). In unstimulated cells, NF-κB is maintained in an inactive state in the cytoplasm by complexing with members of the IκB inhibitory protein family, including IκB-α and IκB-β (Miyamato and Verma, 1995). The interaction between NF-κB and IκB-α masks NF-κB's nuclear localization signal and inhibits the DNA binding activity of NF-κB (Baeuerle and Baltimore, 1996; Miyamato and Verma, 1995). The inducible phosphorylation of serines 32 and 36 of IκB-α (Traencker *et al.*, 1995), by the recently cloned IκB-α kinase, IKKα (Mercurio *et al.*, 1997; Zandi *et al.*, 1998) stimulates the ubiquitination of IκB-α. Ubiquitinated IκB-α is degraded by the 26S proteasome complex (Traencker *et al.*, 1995). NF-κB is then translocated to the nucleus and activates transcription of a variety of genes, including cytokines, cell cycle regulatory proteins, members of the IκB and Rel protein family as well as anti-apoptotic proteins (Baeuerle and Baltimore, 1996; Miyamato and Verma, 1995; Wang *et al.*, 1998; Wu *et al.*, 1998).

The activation of NF-κB is associated with decreased levels of apoptosis. Cells in which NF-κB activation is inhibited by a dominant negative IκB-α protein exhibit increased levels of apoptosis following exposure to DNA damage or the cytokine TNFα (Tumor Necrosis Factor-α; Wang *et al.*, 1996). Transgenic mice lacking the p65 sub-unit of NF-κB exhibit increased levels of apoptosis, and fibroblasts from these mice are more sensitive to TNFα induced cell death than normal cells (Beg and Baltimore, 1996). NF-κB can also activate the transcription of genes which suppress apoptosis, through the regulation of caspase activity (Beg and Baltimore, 1996; Wang *et al.*, 1998; Wu *et al.*, 1998). The activation of NF-κB by various agents, including DNA damage, therefore leads to the transcriptional activation of genes that suppress apoptosis (Wang *et al.*, 1996, 1998; Wu *et al.*, 1998). As a consequence of this, inhibition of NF-κB activation leads to increased cell death.

Prostate cancer is the most commonly diagnosed cancer in men. During the early stages of growth, prostate cancer cells are androgen dependent, and tumor growth can be controlled by anti-androgens. However, tumors eventually become unresponsive to anti-androgen therapy and the tumors progress. In this study, we have examined the NF-κB status of a number of well-characterized prostate cancer cell lines that differ in androgen sensitivity. We found that NF-κB levels were constitutively activated in the hormone independent prostate cell lines PC-3 and DU-145, but not in the hormone responsive LNCaP cells. Further, PC-3 cells showed constitutive activation of IKKα, the kinase responsible for phosphorylation of IκB-α and activation of NF-κB. The NSAID (Non-Steroidal Anti-Inflammatory Drug) ibuprofen inhibits the constitutive activation of NF-κB and IKKα in human

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prostate cell lines, although ibuprofen did not directly inhibit IKKα.

The NF-κB complex consists of homo- or heterodimers between Rel family members, including the widely expressed p65/RelA protein (Baeuerle and Baltimore, 1996; Miyamato and Verma, 1995). Analysis of NF-κB in PC-3 cells by EMSA (Electrophoretic Mobility Shift Assay) revealed multiple DNA-protein complexes (Figure 1a). Antibodies to p65/RelA supershifted the upper, slowly migrating complex. Antibodies to p50 supershifted an additional band below the p65 complex, whereas non-specific antibodies (NS) were without effect (Figure 1a; right). The faster migrating bands were unaffected by either anti-p65 or p50 antibodies (Figure 1a). The upper, slower migrating bands (indicated by the bracket in Figure 1a) are therefore likely to represent NF-κB complexes, whereas the lower bands represent non-specific complexes. Similar levels of p65 and p50 were observed in DU-145 and LNCaP prostate cancer cells (data not shown). In Figure 1a, untreated PC-3 cells displayed elevated levels of NF-κB DNA binding activity, suggesting that NF-κB was already activated in these cells. This is in contrast to other cell types, where NF-κB is inactive unless exposed to cytokines such as TNFα or to stresses such as DNA damage (Baeuerle and Baltimore, 1996; Basu *et al.*, 1998; Miyamato and Verma, 1995). When PC-3 cells were exposed to Ionizing Radiation (IR) or TNFα, no further increase in NF-κB DNA-binding activity was seen (Figure 1a). Since PC-3 cells express significant numbers of TNFα receptors (Nakajima *et al.*, 1996), this implies that NF-κB is fully activated in PC-3 cells.

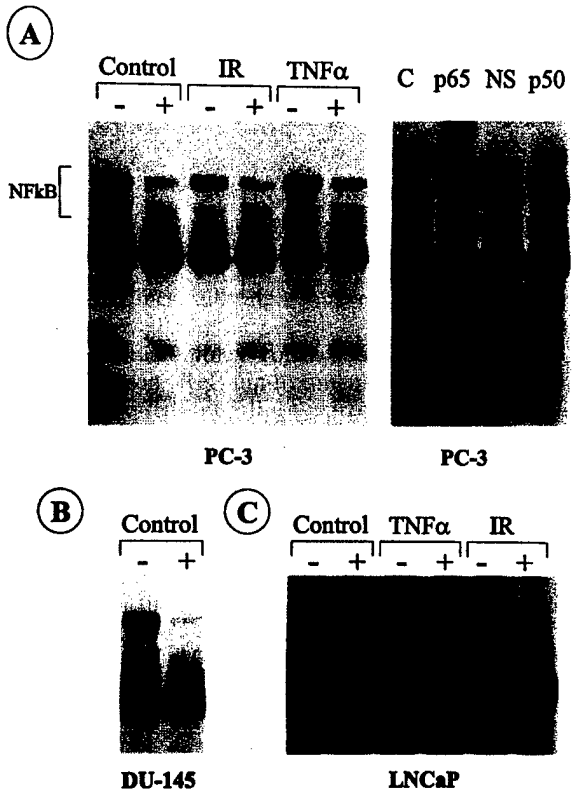

Figure 1 NF-κB DNA binding activity in PC-3, DU-145 and LNCaP cells. (**a**) *Left hand panel*: Cells were incubated for 60 min in buffer (−) or 2 mM ibuprofen (+) and then either untreated (Control), Irradiated (IR, 10 Gy) or exposed to TNFα (10 ng/ml). Cell extracts were prepared 1 h later and EMSA carried out as described below. Position of NF-κB complex are indicated. *Right hand panel*: PC-3 cell extracts were incubated for 20 min with buffer (C), antibodies (1 μg) to p65, p50 or the transcription factor IRF-1 (NS). NF-κB DNA binding activity was then assessed by EMSA. (**b**) DU-145 cells were incubated in buffer (−) or ibuprofen (2 mM; +) and EMSA analysis carried. (**c**) LNCaP cells were incubated for 60 min in buffer (−) or 2 mM ibuprofen (+). Cells were then either untreated (Control), Irradiated (IR) or exposed to TNFα (10 ng/ml) and cell extracts prepared as described below. PC-3, DU-145 and LNCaP cells were maintained as previously described (Palayoor *et al.*, 1998) and treated with ibuprofen in buffer containing HEPES pH 6.8 (20 mM), KCl (120 mM), Glucose (5.5 mM), $CaCl_2$, (1.8 mM) and $MgSO_4$ (1 mM). EMSA. Cell lysates were prepared as described (Basu *et al.*, 1998). EMSA reactions contained: Cell lysate (10 μg), [^{32}P]-NF-κB consensus oligonucleotide (AGTTGAGGGGACTTTCCCAGGC: 0.5 ng), BSA (20 μg), pdI-dC (2 μg), Buffer D+ (2 μl), Buffer F (4 μl) and DTT (1 mM) in 20 μl. Buffer D+: (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40). Buffer F: (20% FICOLL 400, 100 mM HEPES pH 7.9, 300 mM KCl)

Since NSAIDS, including ibuprofen, inhibit cyclooxygenases, suppressing prostaglandin production (DeWitt and Smith, 1995). NSAIDS also inhibit the activation of NF-κB (Kopp and Gosh, 1994), are effective suppressors of tumor growth in human colon (DeWitt and Smith, 1995) and can sensitize prostate cells to Ionizing Radiation (Palayoor *et al.*, 1998; Teicher *et al.*, 1996). We used ibuprofen to examine the mechanism of NF-κB activation in prostate tumor cells. When PC-3 cells were exposed to ibuprofen, the levels of constitutive NF-κB binding activity in PC-3 cells was decreased (Figure 1a, +), without affecting the binding of non-specific proteins to be NF-κB consensus oligonucleotide. Ibuprofen also inhibited NF-κB activity in irradiated or TNFα treated PC-3 cells (Figure 1a). Similar results were seen with DU-145 cells, which displayed constitutive activation of NF-κB that was blocked by ibuprofen (Figure 1b). We next examined the NF-κB status of the androgen responsive prostate cell line LNCaP. LNCaP cells had no detectable basal levels of NF-κB, but showed strong activation of NF-κB by both TNFα and Ionizing Radiation (Figure 1c). Ibuprofen inhibited the activation of NF-κB by both TNFα and Ionizing Radiation in LNCaP cells (Figure 1c).

To control for the specificity of ibuprofen towards NF-κB, we also examined the effect of ibuprofen on the DNA-binding activity of the Oct-1 transcription factor. Oct-1 is a ubiquitously expressed transcription factor which binds to the octamer motif (Latchman, 1999). We examined the effect of ibuprofen on the binding of Oct-1 to its consensus recognition site. In Figure 2a, PC-3 cells displayed a single strong DNA binding activity. This binding activity was not competed by excess unlabeled octamer oligonucleotide with a mutation in the octamer binding site (Figure 2a, mutant). Addition of unlabeled octamer oligonucleotide effectively competed for binding (Figure 2a, self). In addition, an Oct-1 antibody supershifted this band whereas a IgG was without effect (Figure 2a, Oct-1 and IgG). This band therefore represents an Oct-1 DNA complex. In both PC-3 and DU-145 cells, Oct-1 binding was not affected by incubation with ibuprofen. LNCaP cells expressed both the Oct-1 protein as well as a second, lower mobility band (Figure 2a).

Neither band was affected by the addition of ibuprofen. The identity of the upper band is unknown. The lower band is super-shifted by the Oct-1 antibody, but the upper band is unaffected by the Oct-1 antibody and is not competed by the mutant octamer oligonucleotide. This band may represent either Oct-2 (which also binds the octamer sequence), or a related member of the Octamer binding protein family (Latchman, 1999). We have also seen that ibuprofen does not affect AP-1 binding (unpublished observation), and previous studies demonstrate that ibuprofen activates the DNA binding activity of the Heat Shock Transcription Factor (Soncin and Calderwood, 1996). These observations indicate that ibuprofen exhibits some specificity towards NF-κB, and does not inhibit the DNA binding activity of other transcription factors. Figure 1 therefore demonstrates that PC-3 and DU-145 cells display high basal levels of NF-κB which were not increased by further stimulation, whereas LNCaP cells have low basal NF-κB activity but display rapid activation following stimulation. However, ibuprofen consistently blocked both constitutive and inducible NF-κB activity in all three cell lines.

The activation of NF-κB normally proceeds through the ubiquitination and degradation of the IκB-α inhibitory protein (Baeuerle and Baltimore, 1996; Miyamato and Verma, 1995). The elevated levels of NF-κB DNA-binding activity seen in PC-3 cells may therefore result from decreased levels of the IκB-α inhibitory protein. To test this hypothesis, PC-3 and LNCaP cell extracts were examined by Western blotting. PC-3 cells contained low levels of IκB-α (Figure 2b), and addition of ibuprofen actually increased the levels of the IκB-α protein (Figure 2b). This is consistent with the decrease in NF-κB DNA binding activity seen in ibuprofen treated PC-3 cells (Figure 1a). TNFα did not alter IκB-α levels in PC-3 cells (Figure 2b). The levels of the IκB-β and p65 proteins in PC-3 cells were essentially unchanged by treatment with either TNFα or ibuprofen. Actin levels (measured by Western blotting; Figure 2b) are shown as a loading control. In contrast, exposure of LNCaP cells to ibuprofen did not alter the levels of IκB-α. TNFα decreased the levels of IκB-α protein in LNCaP cells, presumably due to increased degradation of IκB-α, and this correlates with the activation of NF-κB DNA-binding activity seen in Figure 1c. Ibuprofen inhibited TNFα induced IκB-α degradation in LNCaP cells (Figure 2b), and blocked the activation of NF-κB (Figure 1c). Again, neither ibuprofen or TNFα altered the levels of IκB-β or p65 protein in LNCaP cells. Figure 2b therefore demonstrates that constitutive activation of NF-κB in PC-3 cells is associated with decreased levels of IκB-α protein. Further, ibuprofen blocks the degradation of IκB-α in both PC-3 and LNCaP cells.

IκB-α levels can be regulated by the inducible phosphorylation of IκB-α, leading to its ubiquitin dependent degradation (Traenckner *et al.*, 1995). IKKα regulates the inducible phosphorylation of IκB-α (Mercurio *et al.*, 1997; Zandi *et al.*, 1998). To determine if IKKα plays a role in the constitutive activation of NF-κB in PC-3 cells, the kinase activity of IKKα immuno-precipitated from PC-3 cells was monitored. PC-3 cells immunoprecipitated with IgG did not phosphorylate an IκB-α fusion protein (Figure 3a). PC-3 cells immunoprecipitated with anti-IKKα antibody phosphorylated IκB-α, but not IκB-β. The IKKα antibody therefore specifically immunoprecipitates a kinase activity which phosphorylates IκB-α but not IκB-β. The GST fusion tag on the substrates was not phosphorylated by IKKα. In Figure 3a (center), IKKα was immunoprecipitated from PC-3 or LNCaP cells stimulated with TNFα. PC-3 cells exhibited high basal levels of IKKα kinase activity, which was not increased by exposure to TNFα (Figure 3a). In contrast, LNCaP cells had low basal levels of IKKα

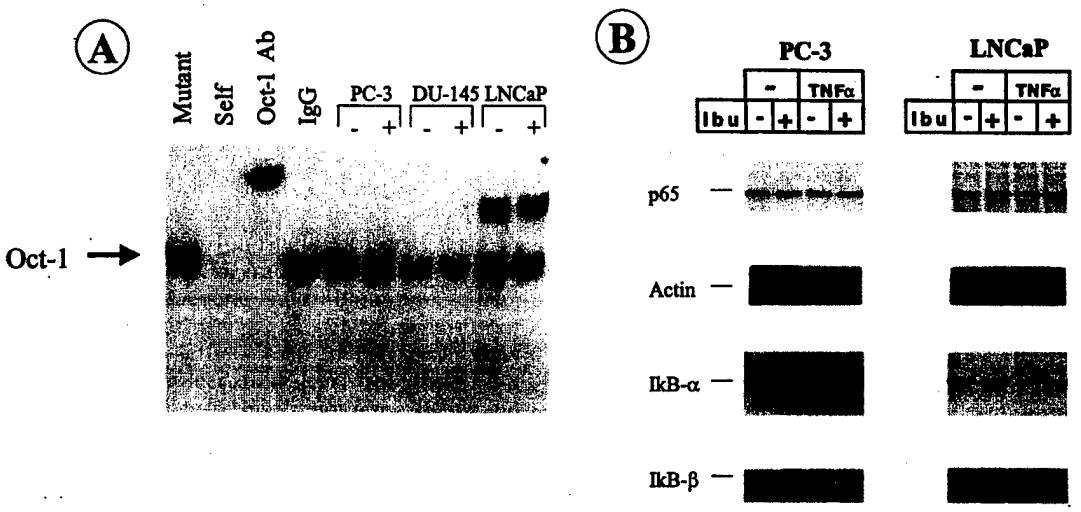

Figure 2 Effect of ibuprofen on DNA-binding activity of Oct-1. (**a**) EMSA was carried out using PC-3, DU-145 or LNCaP cell extracts prepared as in Figure 1 and an oligonucleotide corresponding to the octamer binding sequence (TGTCGAATGCAAATCACTAGAA; 1 ng per reaction). For PC-3 cells, the binding reactions were supplemented with: mutant, 20-fold excess of the mutated octamer oligonucleotide TGTCGAATGCAAGCCACTAGAA; Self, 20-fold excess of unlabeled octamer oligonucleotide; Oct-1 Ab, 1 μg of Oct-1 Antibody; NS, 1 μg of IgG. Control (−) and ibuprofen (+) treated cells are indicated. (**b**) PC-3 or LNCaP cells were untreated or exposed to TNFα (10 ng/ml) in the absence (−) or presence (+) of ibuprofen (2 mM). Cell lysates were prepared as in Figure 1 and 18 μg of protein examined by Western blotting using antibodies specific for p65, actin, IκB-α or IκB-β

kinase activity which were stimulated by TNFα. Western blotting of PC-3 and LNCaP cells indicates that both cell lines expressed similar levels of IKKα protein (Figure 2a, right). p65 levels are shown for comparison. The data are consistent with IKKα being constitutively active in PC-3 cells, but requiring stimulation by TNFα for activation in LNCaP cells.

To determine the mechanism by which ibuprofen inhibits NF-κB DNA-binding activity we examined if IKKα was the target for ibuprofen. PC-3 cells were incubated in ibuprofen and NF-κB DNA binding activity (Figure 3b) and IKKα kinase activity measured (Figure 3c). NF-κB DNA binding activity in PC-3 cells was maximally inhibited between 1–2 mM ibuprofen (Figure 3b). Similarly, constitutive IKKα activity in PC-3 cells was inhibited between 1–2 mM ibuprofen (Figure 3c). In LNCaP cells, TNFα stimulated IKKα activity (Figure 3c), and this activity was also inhibited at 2 mM ibuprofen. Ibuprofen can therefore inhibit the endogenous IKKα kinase activity in both PC-3 and LNCaP cells. To determine if ibuprofen directly inhibits IKKα, IKKα was immunoprecipitated from PC-3 cells. The immunoprecipitated IKKα was then incubated directly with increasing concentrations of ibuprofen prior to the measurement of kinase activity. Under these conditions, we were unable to detect inhibition of IKKα by ibuprofen *in vitro* (Figure 3d). This indicates that ibuprofen may inhibit an upstream regulator of IKKα. Interestingly, low levels of ibuprofen-independent IKKα kinase

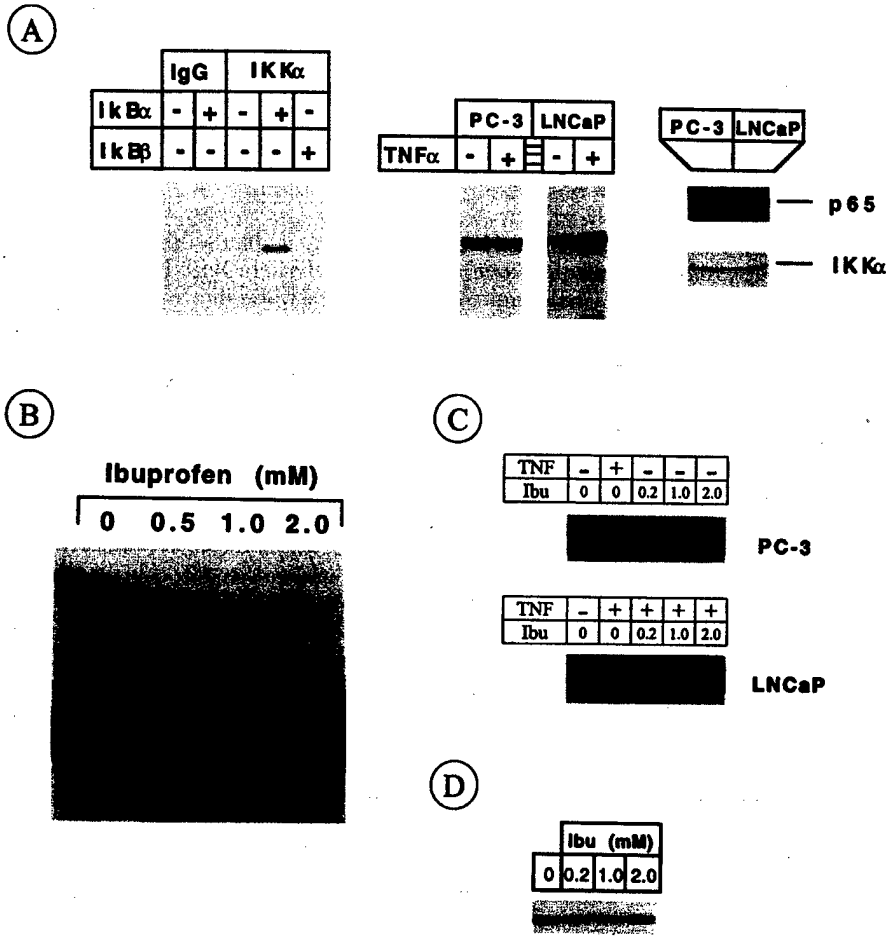

Figure 3 Effect of ibuprofen on the kinase activity of IKKα. (**a**) *Left*. Immunokinase assays were carried out using PC-3 cell lysates immunoprecipitated with IgG or anti-IKKα antibody and with recombinant IκB-α or IκB-β protein as substrate. *Center*. IKKα was immunoprecipitated from untreated or TNFα treated PC-3 or LNCaP cells and IKKα activity assessed using IκB-α as substrate. *Right*. PC-3 or LNCaP cells were examined by Western blotting for p65 or IKKα protein levels. (**b**) PC-3 cells were exposed to increasing concentrations of ibuprofen for 2 h and the levels of NF-κB DNA binding activity measured by EMSA. (**c**) PC-3 or LNCaP cells were incubated in the absence (−) or presence (+) of TNFα for 15 min in the presence or absence of the indicated concentration of ibuprofen. IKKα kinase activity was then determined by immunokinase assay. (**d**) Cell extracts from untreated PC-3 cells were immunoprecipitated with anti-IKKα antibody, washed twice in kinase buffer and then incubated for 15 min in kinase buffer containing the indicated concentrations of ibuprofen. ATP (5 μM, ^{32}P-ATP (10 μCi) and IκB-α (0.7 μg) were then added and immunokinase activity measured. Kinase assays. Cells were lyzed in buffer A (20 mM Tris, pH 7.2; 0.5 M NaCl; 0.5% NP40; 1 mM EDTA; 1 mM DTT; 1 mM PMSF) and the supernatants cleared by centrifugation at 15 kg for 10 min, 1.5 μg of IgG or anti-IKKα antibody were prebound to Protein-A/G-Agarose at 4°C for 17 h. Cell extracts (200 μg) were incubated with the immobilized antibody for 2 h at 4°C, washed four times in 1 ml of buffer A, and twice in kinase buffer (8 mM MOPS, pH 7.2; 10 mM $MgCl_2$; 0.2 mM EDTA). Excess liquid was removed with a hypodermic needle. 30 μl of kinase buffer containing ATP (5 μM), ^{32}P-ATP (10 μCi) and IκB-α (0.7 μg) was added to each tube and allowed to incubate for 10 min at room temperature. Reactions were terminated by the addition of 11 μl of 4 × SDS sample buffer and the phosphorylated proteins separated by SDS–PAGE. GST-IκB-α and GST-IκB-β were prepared as previously described by us (Basu *et al*., 1998)

activity were always detected at doses of ibuprofen of 2 mM (Figure 3c). This may represent the basal IKKα activity in unstimulated cells, and would be unaffected by inhibition of an upstream regulator by ibuprofen.

The results demonstrate that PC-3 and DU-145 prostate cancer cells exhibit constitutive activation of the NF-κB transcription factor, whereas LNCaP cells exhibit TNFα-induced NF-κB activation. Tumor cells derived from breast cancer (Nakshatri *et al.*, 1997) or Hodgkin's disease (Krappmann *et al.*, 1999) also exhibit constitutive activation of NF-κB. In Hodgkin's disease, NF-κB activation is associated with activation of IKKα and decreased levels of IκB-α (Krappmann *et al.*, 1999). Elevated levels of the p65 sub-unit of NF-κB can increase IκB-α levels (Perez *et al.*, 1995) due to transcriptional activation of IκB-α by p65 (Sun *et al.*, 1993). However, the ability of ibuprofen to suppress NF-κB activation is associated with an increase in IκB-α levels and decreased IKKα activity in the absence of any detectable change in p65 levels. We interpret this as an indication that p65 levels themselves do not directly contribute to the activation of NF-κB in PC-3 and DU-145 cells. Instead, the constitutive activation of NF-κB is a consequence of IKKα activation, which in turn phosphorylates IκB-α, triggering its degradation and allowing p65 to accumulate in the nucleus. Our results are the first demonstration that NF-κB is activated in androgen independent prostate tumor cell lines, but not in an androgen-responsive tumor cell line. Analysis of additional prostate tumor cell lines will be needed to determine if NF-κB activation is a common event in androgen independent cells.

The mechanism by which constitutive NF-κB activation occurs is unclear. Both PC-3 and DU-145 cells secrete large amounts of interleukin-6 and other cytokines (Nakajima *et al.*, 1996), whereas LNCaP cells secrete much lower levels. Cytokines can activate NF-κB, and NF-κB can itself activate the transcription of cytokines such as interleukin-6 (Baeuerle and Baltimore, 1996). Chronic autocrine stimulation of the NF-κB may therefore account for constitutive NF-κB activation in PC-3 and DU-145 cells. However, whether the high levels of interleukin-6 (or other factors) secreted by PC-3 and DU-145 cells are the cause or consequence of NF-κB activation is not known. An alternative mechanism for constitutive activation of NF-κB in PC-3 cells is activation of an internal signal transduction pathway, perhaps due to mutation or inappropriate expression of regulatory proteins in these tumor cells. For example, over-expression of the anti-apoptotic protein bcl-2 protein can activate NF-κB and suppress apoptosis (de Moissac *et al.*, 1998), and many advanced prostate tumors express bcl-2 (Apakama *et al.*, 1996). The constitutive activation of NF-κB in prostate tumor cells may have a number of consequences. These include increased production of NF-κB regulated cytokines as well as suppression of the apoptotic response. NF-κB can repress transcription of the Androgen Receptor (Supakar *et al.*, 1995) and can bind to and inhibit Androgen Receptor function (Palvimo *et al.*, 1996). In addition, neither PC-3 nor DU-145 cells express detectable levels of the Androgen Receptor, whereas LNCaP cells contain high levels of the receptor (Tilley *et al.*, 1990). This suggests that constitutive activation of NF-κB in PC-3 and DU-145 cells may also inhibit expression of the Androgen Receptor. The activation of NF-κB may therefore contribute to the emergence of androgen-independent prostate tumor cells and the development of the malignant phenotype.

IKKα was inhibited in ibuprofen treated cells but this was not due to direct inhibition of IKKα by ibuprofen. This implies that ibuprofen inhibits an upstream regulator of IKKα. Ibuprofen also inhibited the activation of NF-κB by both TNFα and DNA damage (Figure 1c). This suggests that ibuprofen inhibits a signaling component which is common to the pathways utilized by TNFα and Ionizing Radiation to activate NF-κB. Several members of the MEKK kinase family have been implicated in the upregulation of the IKKα enzyme complex, including NIK (Ling *et al.*, 1998) and MEKK1 (Lee *et al.*, 1998). IKKα is part of a large multiprotein complex including IKKα, IKKβ, NIK, IκB-α and -β, NF-κB sub-units as well as other unidentified components (Cohen *et al.*, 1998). Ibuprofen may be an inhibitor of the upstream regulator of this complex, or a generic inhibitor of all MEKK kinases. Other NSAIDS, such as aspirin, can inhibit IKKβ, the kinase which phosphorylates IκB-β (Yin *et al.*, 1998). However, we have been unable to detect any effect of ibuprofen on IκB-β protein levels (Figure 2b), although it is possible that ibuprofen may also inhibit IKKβ.

Ibuprofen alone does not affect DU-145 or LNCaP tumor growth in mice (Teicher *et al.*, 1996). In culture, 1 mM ibuprofen does not cause significant growth delay of PC-3 or DU-145 cells, although higher doses (2 mM and above) can cause growth delay and increase the apoptotic rate (Palayoor *et al.*, 1998). However, ibuprofen can sensitize DU-145, PC-3 and LNCaP cells to radiation both in culture and in animal tumor models (Palayoor *et al.*, 1998; Teicher *et al.*, 1996). Ibuprofen therefore only enhances cell death in combination with an associated genotoxic event. The serum levels of ibuprofen achieved clinically are of the order of 0.2 mM (Laska *et al.*, 1986), although higher levels can be tolerated acutely. The inhibition of IKKα seen here *in vitro* at 1 mM ibuprofen (Figure 3c) may therefore be achievable *in vivo*.

NSAIDS inhibit COX-1 and COX-2, the enzymes responsible for the synthesis of prostaglandins. NSAIDS are also effective suppressors of tumor growth in human colon (DeWitt and Smitt, 1995). However, the concentrations of NSAIDs required to inhibit prostaglandin synthesis are much lower than those required to exert anti-tumor effects. Whether NSAIDS suppress tumor growth through inhibition of prostaglandin synthesis is therefore unclear. NF-κB can also activate the transcription of a variety of anti-apoptotic genes, including TRAF1 and -2, cIAP-1 and -2 and IEX-1L, leading to suppression of apoptosis, perhaps by modulation of caspase activity (Wang *et al.*, 1998; Wu *et al.*, 1998). Inhibition of NF-κB by genetic methods is associated with increased apoptotic cell death following exposure to DNA damage or to TNFα (Beg and Baltimore, 1996; Wang *et al.*, 1996). In addition, we have shown that ibuprofen sensitizes prostate tumor cells PC-3 and DU-145 to radiation therapy in both tissue culture and animal models (Palayoor *et al.*, 1998; Teicher *et al.*, 1996). The

constitutive activation of NF-κB in prostate tumor cells may result in the expression of high levels of anti-apoptotic proteins. This, in turn, may suppress the normal apoptotic response and allow the cells to survive DNA damage, decreasing the effectiveness of anti-tumor therapy. The inhibition of NF-κB activation in these prostate tumor cells by ibuprofen may therefore contribute to increased cell death through inhibition of transcription of anti-apoptotic genes. NSAIDs may prove to be valuable drugs for use in the treatment of tumors, and may provide a starting point for the rational design of agents which specifically inhibit the activation of NF-κB. Further, the ability of NSAIDS to act as suppressor of tumor growth may be, in part, related to their ability to inactivate NF-κB.

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Ibuprofen-mediated reduction of hypoxia-inducible factors HIF-1α and HIF-2α in prostate cancer cells[1]

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to have anti-angiogenic properties. Because the hypoxia-inducible transcription factors are key regulators of tumor angiogenesis, we determined the effect of the NSAID ibuprofen on HIF-1α and HIF-2α in PC3 and DU-145 human prostate cancer cells. Ibuprofen treatment rapidly reduced HIF proteins under normoxic and hypoxic conditions with a subsequent reduction in the HIF-regulated gene products VEGF and Glut-1. These data suggest that a therapeutically feasible approach to targeting HIF is through the use of NSAIDs. The concentration of NSAIDs required to inhibit HIF and HIF-regulated proteins *in vitro* was higher than the concentration required to inhibit prostaglandin synthesis suggesting that the inhibition was COX-2 independent.

Introduction

There is increasing evidence suggesting that non-steroidal anti-inflammatory drugs (NSAIDs) [3] may have potential use as anti-cancer agents, either alone or in combination with other cancer therapies (1-8). Whereas NSAID treatment can result in tumor cell death (3, 9, 10), a potentially critical effect of these agents is the inhibition of angiogenesis (1, 4, 5, 11-17). The specific mechanism responsible for the antiangiogenic actions of NSAIDs has not defined. A number of studies have suggested that the anti-angiogenic effect of NSAIDs can be attributed to the specific inhibition of the COX-2 enzyme (11-12, 14-16). However, even the tumor cells that lack COX-1 and COX-2 produce proangiogenic factors and stimulate angiogenesis (12) suggesting that in addition to COX-2, NSAIDs may target other angiogenic pathways as well

(12, 13, 17). A major initiator of angiogenesis is hypoxia, which induces a wide variety of genes through the activation of transcription factor HIF-1α, of member of the bHLH-PAS gene family (18-20). Under normoxic conditions HIF-1α interacts with tumor suppressor pVHL and is rapidly degraded via ubiquitin-dependent proteasome pathway (21-24). Hypoxia induces a rapid redox-sensitive increase in HIF-1α protein stability and transcriptional activity (25-27) resulting in the activation of target genes involved in erythropoiesis, glycolysis and angiogenesis (20, 28-31). In addition to the genes required for metabolic adaptation to hypoxia, HIF-1α also enhances the expression of genes coding for growth factors, growth factor receptors, components of the apoptotic pathway and cell cycle regulators (20, 31, 32). HIF-2α, a recently cloned member of bHLH family, shows close sequence homology and similar pharmacological and regulatory properties as HIF-1α (33-35). Because HIF-1α and 2α can regulate the expression of genes not only involved in angiogenesis but also those that contribute to tumor cell survival and aggressiveness, these hypoxia inducible factors have been suggested as potential targets for anti-neoplastic therapy (20). Because the majority of solid tumors contain regions of hypoxia, hypoxia promotes angiogenesis and NSAIDs inhibit angiogenesis, we hypothesized that NSAIDs inhibit the HIF transcription factors. In the study presented here we show that treatment of PC3 and DU-145 prostate carcinoma cell lines with the NSAID ibuprofen reduced HIF levels under normoxic and hypoxic conditions with a subsequent reduction in the HIF-regulated gene products VEGF and Glut-1. PC3 cells expressed COX-2 protein while DU-145 cells did not. These data suggest that the anti-angiogenic actions of NSAIDs may be mediated at least in part via a decrease in these hypoxia-inducible factors.

Materials and Methods

Materials: PC3 and DU-145 human prostate carcinoma cells from ATCC were maintained in RPMI 1640 media supplemented with 10%FBS, glutamine and antibiotics. Anti-HIF-1α monoclonal antibody was purchased from Transduction Labs, anti-HIF-2α polyclonal antibody from Novus Biologicals, anti-Glut-1 antibody from Alpha Diagnostics and anti COX-2 and anti-topoisomerase-1 polyclonal antibodies were from Santa Cruz Biotechnology. Anti-actin antibody was purchased from Chemion. Ibuprofen, diclofenac, ketorolac and indomethacin were purchased from Sigma Chemicals. Ibuprofen (I 1892, Sigma Chemicals) was dissolved in water (100mM) and filter-sterilized prior to addition to culture media. NS398 was purchased from Cayman Chemical and prepared as a 100mM stock in DMSO.

Cell treatment: To induce hypoxia, cells were plated in 70cm^2 glass flasks and used when approximately 70% confluent. Prior to gas equilibration the media was removed and replaced with fresh media without or with ibuprofen. After 30 min, flasks were tightly sealed with rubber stopper and hypoxia was induced by gassing with a mixture of 95% nitrogen and 5% CO2 for 1 h (36). After 1 h of gassing, the cells were returned to the incubator in the rubber-stopper sealed flasks. Under these conditions, hypoxia was maintained for the duration of the experiment.

Western Blot Analysis: Whole-cell extracts were prepared in lysis buffer containing 50mM Tris pH 7.5, 150mM NaCl, 1% Igepal, 0.1% SDS, 1mM EDTA, 600μM sodium orthovanadate, 50mM sodium fluoride, "complete" protease inhibitor (Roche Diagnostics Corp), 1mM PMSF and 1mM DTT. After incubating on ice for 20min, cell extracts were centrifuged at 20,000 x g for 10 min and supernatants were collected. Protein concentrations were determined by Bio-Rad D_C protein assay. Sixty μg protein from normoxic samples and 30μg protein from

hypoxic samples were separated on 6% gels for HIF-1α and HIF-2α analyses and 10% gels were used for Glut-1 and COX-2.

VEGF levels: Cells were plated in glass flasks ($15cm^2$) in 2ml media, treated with 2mM Ibu and subjected to hypoxia as described earlier. Media was collected, centrifuged at 750 x g for 5 min, and stored at –70°C. The concentration of the secreted $VEGF_{165}$ isoform in the media was determined with an ELISA kit (R&D Systems) according to the manufacturer's instructions.

Results

In the PC3 and DU-145 cell lines, the levels of the HIF-1α protein detected under normoxic conditions were dependent on the length of time in culture media (Fig 1A). After 24-48h in media containing 10% FBS, HIF-1α protein was readily detectable in both cell lines (Fig 1A, left panels). However, HIF-1α levels were rapidly reduced when the conditioned media was replaced with fresh media (Fig 1 A, right panels); over the next 24h HIF-1α levels then returned to those observed before replacement of the conditioned media. These data suggest that whereas serum factors may be required for the increased HIF-1α levels under normoxic conditions as reported by others (37), their maintenance results from the presence of a factor (s) provided by the cells themselves. Exposure of cell lines in conditioned media to 2 mM Ibu resulted in a rapid reduction in HIF-1α levels reaching undetectable levels by 60 min (Fig 1A, left panels), which were then maintained at an undetectable level for 24 h. When Ibu was added at the time of fresh media, the increase in HIF-1α levels that occurred by 24 h was prevented (Fig 1A, right panels). As shown in figure 1B reductions in HIF-1α levels were detectable by 10 and 30 minutes in PC3 and DU-145 cells, respectively. The dose response determined at 60 min revealed significant decreases after 1 mM in PC3 cells and 0.5 mM in DU-145 cells. These data

thus indicate that Ibu reduced constitutive HIF-1α levels and the accumulation of HIF-1α following addition of fresh media.

To determine the effects of Ibu on hypoxia-induced HIF-1α protein, PC3 and DU-145 cells were grown in glass flasks and gassed with 95%/5% N2/CO2 for 1 h. As shown in figure 2A, as compared to normoxic conditions, HIF-1α levels were significantly increased in both cell lines after the 1h hypoxia gassing procedure. HIF-1α remained elevated after 4 h of exposure to hypoxia decreasing by 24h (Fig. 2B). Addition of 2mM Ibu significantly reduced HIF-1α levels as determined after the 1 h gassing procedure (Fig. 2A). After 4 h of hypoxia, the inhibitory effect of Ibu, although present, was only about 50% as compared to HIF-1α in cells exposed to hypoxia only. After 24 h of hypoxia, HIF-1α decreased in the hypoxia only cells and Ibu had essentially no effect in PC3 cells and actually increased HIF-1α levels in the hypoxic DU-145 cells. These data indicate that Ibu reduces the initial hypoxia-induced increase in HIF-1α.

In addition to HIF-1α, a critical transcription factor mediating hypoxia-induced changes in gene expression is HIF-2α. To determine the effects of Ibu on HIF-2α levels, PC3 and Du-145 cells were exposed to this NSAID under normoxic and hypoxic condition (Fig. 2C). As for HIF-1α, in cells maintained in growth media for at least 24 h, HIF-2α was detectable under normoxic conditions in both cell lines. After exposure to hypoxic conditions there was a significant increase in HIF-2α in both cell lines, which was maintained for at least 24 h. Addition of Ibu decreased HIF-2α in cells under normoxic and hypoxic conditions. However, in contrast to the effects of Ibu on HIF-1α levels (Fig. 2B), the reductions in HIF-2α were maintained for the entire 24 h hypoxia exposure period.

The data presented above indicate that Ibu reduces the levels of the HIF-1α and HIF-2α transcription factors. To gain insight into the potential physiological significance of this effect, the expression of two gene products that are induced by hypoxia and regulated by HIF were determined. VEGF is a critical angiogenic factor and is well established to be increased under hypoxia and at least in part regulated by HIF (20, 31). Therefore, the effects of Ibu on secreted VEGF levels were determined under normoxic and hypoxic conditions. VEGF was detectable in the media from normoxic PC3 cells and was dramatically increased by 24 h of hypoxia (Fig. 3A). Whereas Ibu had little effect on VEGF levels under normoxic conditions after 24 h, the VEGF levels after 24 h of hypoxia in the presence of 2mM Ibu were reduced by approximately 50%. Similar effects were observed for DU-145 cells. The VEGF concentrations (mean ± SD, n=3) in media from untreated and ibuprofen-treated normoxic DU-145 cells at 24h were 4.81 ± 0.82 ng/10^6 cells and 4.09 ± 0.81 ng/10^6 cells, respectively. Hypoxia increased the VEGF concentration to 7.72 ± 0.60 ng/10^6 cells; ibuprofen treatment of hypoxic DU-145 cells resulted in a reduction in VEGF levels to 3.97 ± 1.43 ng/10^6 cells.

An additional protein induced by hypoxia and regulated by the HIFs is Glut-1. In western blots of cell extracts Glut-1 protein migrated as a broad multi-band complex between approximately 45-60kDa (Fig. 3B), which is consistent with previous reports (38). Glut-1 protein levels were increased in PC3 and DU-145 cells after 24h of hypoxia (Fig. 3B). Whereas Ibu had essentially no effect on Glut-1 levels under normoxic conditions, 2mM Ibu treatment under hypoxic conditions resulted in a significant reduction in Glut-1 in both cell lines. These data indicate that Ibu also decreases the levels of proteins corresponding to HIF-regulated genes.

To determine whether the inhibition of HIF-1α was specific to Ibu or it was a feature shared by other NSAIDs as well, PC3 and DU-145 cells were treated with other nonspecific

NSAIDs diclofenac, ketorolac and indomethacin. These NSAIDs inhibited HIF-1α (Fig. 4A) as well as HIF-2α (data not shown) under normoxic condition and also under hypoxic condition (Fig. 4B) to varying degrees at the concentrations studied.

Treatment of PC3 and DU-145 cells with COX-2 specific NSAID NS398 at 100μM concentration under the same experimental conditions (10% serum media) failed to inhibit HIF-1α (Fig. 5A). When cell were treated with NS398 in 0.1% serum media slight inhibition of HIF-1α was observed (data not shown), so we treated PC3 cells in 0.1% serum media with 100μM NS398 for 1h and then exposed to hypoxia. There was essentially no effect on hypoxic accumulation of HIF-1α with NS398 at 1h (Fig. 5B) as compared to the significant inhibition seen with 2mM Ibu (Fig. 2A, 4B) at this time point. Furthermore, treatment with 100μM NS398 failed to inhibit the HIF-regulated proteins VEGF (Fig. 5C) and Glut-1 (Fig. 5D) at 24h.

To determine the concentration of Ibu required to inhibit HIF-1α and HIF-regulated proteins VEGF and Glut-1 under hypoxia, PC3 and DU-145 cells were treated with different concentrations of Ibu and exposed to hypoxia. HIF-1α levels were analyzed at 1h and VEGF and Glut-1 were analyzed at 24h. Inhibition of HIF-1α was not seen in cells that were treated with lower concentrations of Ibu (0.5-1mM) (Fig. 6A). At these concentrations, Ibu did not inhibit VEGF (Fig. 6B) and Glut-1 (Fig. 6C) in PC3 and DU-145 cells. Reduction in VEGF and Glut-1 was seen only at a concentration at which HIF-1α was inhibited.

The anti-angiogenic effect of NSAIDs such as Ibu has been suggested to involve the COX-2 (11, 12, 14-16). To determine whether the Ibu-mediated reduction in HIFs is dependent on COX-2, the levels of this protein were determined in the two prostate carcinoma cell lines (Fig. 7). PC3 cells expressed similar levels of COX-2 under normoxic and hypoxic conditions. Ibu treatment resulted in an increase in COX-2 levels under both conditions, which is consistent

with previous results from a number of NSAIDs (9, 39). However, with respect to a role for COX-2 in the Ibu-mediated reduction in HIF-1α and HIF-2α levels, the most significant data was obtained from DU-145 cells. In this cell line COX-2 protein was not detected and no increase was detected after hypoxia or Ibu treatment. These data suggest that COX-2 does not mediate the effects of Ibu on HIF protein levels.

Discussion

The HIF proteins were initially associated with expression under hypoxic conditions. However, the data presented here indicate that after growth in unchanged media under normoxic conditions for 24 h HIF-1α and HIF-2α are readily detectable in the prostate carcinoma cell lines PC3 and DU-145. The detection of HIF-1α in prostate cancer cells under normoxic condition was first reported by Zhong et al (40), which suggested a decoupling of HIF-1α protein expression from O_2 tension. More recently HIF-1α expression has been detected in a variety of cancer cell lines under non-hypoxic conditions (30, 33, 37, 41). In a recent study by Zhong et al cell cultures were serum-starved; the addition of 10% serum or EGF was then shown to increase HIF-1α levels (37). These results suggested that, in addition to hypoxia, growth factors play a role in increasing HIF-1α levels. In the studies presented here, cultures grown in media containing 10% FBS for at least 24h expressed readily detectable levels of HIF-1α. However, when the conditioned media was removed and fresh media added there was a rapid and almost complete loss of HIF-1α protein, which returned to initial levels by 24 h. These data suggest that changing the media removes an essential factor(s) necessary to maintain HIF-1α levels under non-hypoxic condition, i.e., those induced by serum factors as shown by Zhong et al (37). The increase of HIF-1α within 24 h of fresh media addition suggests that the factor originates from

the cells. Indeed, recent studies have shown that HIF-1α can be induced by various growth factors (20, 31, 42) and, in turn, induces expression of genes coding for growth factor receptors and binding proteins (32, 42). The expression of HIF-1α in tumor cells may thus generate an autocrine loop (32) maintaining its own expression in cancer cells.

The data presented here show that Ibu reduces HIF-1α levels in cells grown under normoxic and hypoxic conditions. However, this reduction was more complete in normoxic cells. Previous studies have reported that the inhibition of HIF-1α expression by inhibition of the PI3 kinase pathway is also more effective under normoxic than under hypoxic conditions (30, 37). These results along with those in the current study suggest that the regulation of HIF-1α operates through more than 1 pathway/process and that to completely limit HIF-1α expression it may be necessary to target more than one regulatory process. HIF-1α levels decreased rapidly following the addition of Ibu. The specific mechanism by which Ibu reduces HIF is currently under investigation; its delineation should provide insight into the fundamental processes regulating the expression of this protein.

In the present study, the increase in HIF-1α was evident at the end of 1h hypoxic gassing period. Thereafter, the protein levels remained high for up to 4-6h of hypoxia and returned to basal levels by 24h. This is in agreement with earlier finding in Hep 3B cells where maximum accumulation of HIF-1 α protein was seen at 4-8h of continuous hypoxia and reduced by 16h. The kinetics of HIF-1 α DNA-binding activity is similar to the induction of HIF-1α protein (18). We observed an increase in HIF-1 regulated gene products VEGF and Glut-1 at 24h subsequent to the upregulation of HIF-1α at 4h. Although HIF-1α was partially inhibited at 4h, Ibu had essentially no effect at 24h of continuous hypoxia in PC3 cells and actually HIF-1 α level was higher compared to the hypoxic control in DU-145 cells. In spite of lack of inhibition of HIF-1α

by Ibu at 24h under hypoxic condition, both VEGF and Glut-1 were inhibited. This apparent discrepancy between the time courses of HIF-1 and VEGF/Glut-1 inhibition after ibu treatment may reflect the different time requirements for the sequential activation of a transcription factor, the subsequent expression of the secondary response gene and the ultimate changes in level of the corresponding protein. Alternatively, the HIF-1 α protein observed at 24h of continuous hypoxia may not be functionally active. It has been suggested that the predominant HIF-1 α protein species accumulating at later time points probably undergo posttranslational modification as they migrate at a higher position in the gel (18). Our data suggests that the reduction in the initial hypoxia-induced increase in HIF-1 α by Ibu is sufficient to reduce the increase in HIF-1-regulated gene products at 24h.

Whereas a number of agents that inhibit signaling pathways have been reported to reduce HIF-1α, the effects of these agents on HIF-2α have not been reported. However, it would appear that if the therapeutic goal were to reduce the expression of genes regulated by hypoxia inducible factors, it would be necessary to target both HIF-1α and HIF-2α. The data presented here clearly illustrate that Ibu targets both proteins. The reduction in HIF-1α and HIF-2α may account for the reduction in hypoxia-induced VEGF and Glut-1 proteins in Ibu treated cells. Thus, decreasing both forms of HIF may contribute to the anti-cancer effects, both in prevention and treatment, of NSAIDs.

Several studies have demonstrated that NSAIDs inhibit tumor growth by inhibiting angiogenesis (1, 4, 5, 43-45). NSAID-mediated inhibition of angiogenesis is generally attributed to the inhibition of COX-2, since NSAIDs are primarily associated with the inhibition of cyclooxygenases (11-16). It is well documented that in addition to COX-2, angiogenesis is also regulated by HIFs under hypoxia by increasing angiogenic factors including VEGF and VEGF

receptor, Flt-1 (17, 20, 28, 31, 46). The inhibition of VEGF by Ibu in the present *in vitro* study appears to be regulated by HIFs. This is supported by following observations. PC3 and DU-145 cell lines used in this study have different COX-2 profiles. PC3 cells expressed COX-2 protein constitutively, whereas DU-145 cells did not contain detectable COX-2 protein. Hypoxia had no effect on COX-2 protein level in both cell lines. On the contrary, hypoxia up regulated HIFs in both cell types with a subsequent increase in VEGF secretion. The inhibition of HIFs by Ibu resulted in the inhibition of VEGF in both cell types. In the absence of inhibition of HIF-1α, there was no inhibition of VEGF and Glut-1 in cells treated with lower concentrations of Ibu or 100μM NS398. Finally, the concentration of Ibu required to inhibit HIF-1α and HIF-regulated gene products, VEGF and GLUT-1, was higher than the concentration that is required to inhibit prostaglandin synthesis. Our data support the recent observations of Jones et al who showed that in gastric microvascular endothelial cells NSAIDs indomethacin and NS398 inhibited hypoxia-induced accumulation of HIF-1α resulting in inhibition of hypoxia-induced VEGF/Flt-1 expression (17). However, in the present study NS398 did not inhibit hypoxic accumulation of HIF-1α and HIF-regulated proteins in prostate cancer cells at the concentration at which NS398 was effective in endothelial cells. This discrepancy could be due to the differences in normal versus cancer cell types.

Inhibition of endothelial cell proliferation, tube formation and VEGF expression is seen at relatively high concentrations of indomethacin (0.25-0.5mM) or NS398 (100μM) (13, 17, 47). Although NSAIDs inhibit prostaglandin synthesis at micromolar concentrations, several other effects of NSAIDs including induction of apoptosis, cytotoxicity, inhibition of various transcription factors and kinases require concentrations that are well above those that inhibit of prostaglandin synthesis (3, 8-10, 13, 39, 47). *In vitro*, even the COX-2 specific inhibitors appear

to have a prostaglandin-dependent and prostaglandin-independent component (9). The *in vivo* inhibition of angiogenesis and tumor growth by some of the COX-2 specific NSAIDs is recently attributed to the NSAID-mediated inhibition of host stromal and angiogenic vasculature COX-2 (15, 16). The significance of inhibition of HIFs and HIF-regulated gene products by NSAIDs needs to be further investigated in *in vivo* models.

HIF-1α has clearly been shown to aid in a cell's ability to adapt to the hypoxic milieu. However, it has also been implicated in tumor cell survival and proliferation (48). Studies have shown that growth of tumors derived from HIF-1α inactivated cells is significantly slower than matched cells containing the functional protein (19, 49). Furthermore, tumors derived from HIF-1α deficient cells were also deficient in expression of VEGF, a critical tumor angiogenic factor (19, 49, 50). Finally, constitutive expression of HIF-1α was reported to render pancreatic cancer cells resistant to apoptosis induced by hypoxia or glucose deprivation (41). Compared to adjacent normal tissues HIF-1α is overexpressed in a majority of the common human cancers evaluated (51). Thus, in recent years HIF-1α has emerged as a potentially important therapeutic target for cancer therapy (20, 52). Strategies suggested for HIF-1α targeting include disruption of the normal coactivational response to hypoxia (53) and the use of decoy oligonucleotides (52). In the present *in vitro* study the inhibition of HIFs by NSAID ibuprofen correlated with the inhibition of HIF-regulated gene products in tumor cells. Our study along with the earlier report by Jones et al (2002) demonstrates HIFs as novel molecular targets for cancer therapy

Foot notes

[3]The abbreviations used are: Ibu, ibuprofen; NSAIDs, non steroidal antiinflammatory drugs; COX-2, cyclooxygenase-2; HIF, hypoxia-inducible factor; bHLH-PAS, basic helix-loop-helix PER ARNT SIM; VHL, von Hippel-Lindau; VEGF, vascular endothelial growth factor; Glut-1, glucose transporter-1; PBS, phosphate buffered saline; FBS, fetal bovine serum.

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FIGURE LEGENDS

Figure 1: Effect of Ibu on HIF-1α levels in PC3 and DU-145 cells under normoxic conditions. A) Ibu (2 mM) was added to cultures maintained in growth media for 24 h (conditioned media, left panels) or immediately after the addition of fresh media (right panels). Western blots are shown for PC3 cells (top panels) and DU-145 cells (bottom panels). (B) HIF-1α levels as a function of time after Ibu addition. Subconfluent PC3 cells (left panel) and DU-145 cells (right panel) were treated with Ibu (2mM) for indicated times and cell extracts prepared for determination of HIF-1α levels. (C) HIF-1α levels as a function of Ibu dose. Subconfluent PC3 cells (left panel) and DU-145 cells (right panel) were treated with the indicated concentrations of Ibu and HIF-1α determined 1 h later.

Figure 2: Influence of hypoxia and Ibu on HIF-1α and HIF-2α protein levels. (A) PC3 (left panel) and DU-145 cells (right panel) were treated with Ibu (2 mM), subjected to 1h hypoxic gassing and HIF-1α levels determined by western analysis. (B) HIF-1α levels in PC3 (top panel) and DU-145 cells (bottom panel) maintained under hypoxic conditions for the indicated time periods with and without Ibu (2 mM). (C) HIF-2α levels in PC3 (top panel) and DU-145 cells (bottom panel) maintained under hypoxic conditions for the indicated time periods with and without Ibu (2 mM).

Figure 3: Effect of Ibu on the expression of HIF regulated proteins. (A) VEGF secretion in PC3 cells. PC3 cells were treated with 2mM Ibu after addition of fresh media and subjected to hypoxia. At 4h and 24h VEGF concentration in the media was determined using an ELISA. This experiment was performed twice and data from a representative experiment is shown. Glut-1 protein levels in (B) PC3 and (C) DU-145 cells. Cells were treated with 2mM Ibu in fresh media, subjected to hypoxia and prepared for western analysis 24h later.

Figure 4: Effect of nonspecific NSAIDs on HIF-1α levels in PC3 and DU-145 cells under A) normoxic and B) hypoxic condition. (A) HIF-1α levels in cells treated with NSAIDs for 1h. Control: (lanes 1, 5), 2mM Ibu: (lanes 2, 6), 0.3mM diclofenac: (lanes 3, 7) and 2mM ketorolac: (lanes 4, 8). (B) HIF-1α levels in cells treated with NSAIDs for 1h followed by 1h of hypoxia. Hypoxia alone: (lanes 1, 6), 2mM Ibu: (lanes 2, 7), 0.3mM diclofenac: (lanes 3, 8), 2mM ketorolac: (lanes 4, 9) and indomethacin: (lanes 5, 10). HIF-1α levels were determined by western analysis.

Figure 5: (A) Effect of COX-2 specific NSAID NS398 on HIF-1α under normoxic condition. DU-145 and PC3 cells were treated with 100μM NS398 in 10% serum media for the times indicated. (B) PC3 cells were treated with 100μM NS398 in 0.1% serum media for 1h and then exposed to hypoxia. HIF-1α levels were analyzed at 1h. (C) VEGF levels (% of control) at 24h in PC3 and DU-145 cells treated with 100μM NS398 and exposed to hypoxia. This experiment was performed twice and data from a representative experiment is shown. (D) Glut-1 levels at 24h in PC3 cells treated with 100μM NS398 in 0.1% serum and exposed to hypoxia.

Figure 6: HIF-1α, VEGF and Glut-1 levels under hypoxic condition as a function of Ibu dose. PC3 and DU-145 cells were treated with the indicated concentrations of Ibu for 1h in fresh media followed by 1h of hypoxic gassing. A) HIF-1α levels were determined by western analysis after 1h of hypoxia. B) VEGF concentration in media at 24h of hypoxia was measured by ELISA. C) Glut-1 levels at 24h of hypoxia were determined by western analysis.

Figure 7: Effect of Ibu and hypoxia on COX-2 protein in (A) PC3 cells and (B) DU-145 cells. Cells were treated with 2mM Ibu in fresh media, subjected to hypoxia and analyzed at 24h after treatment.

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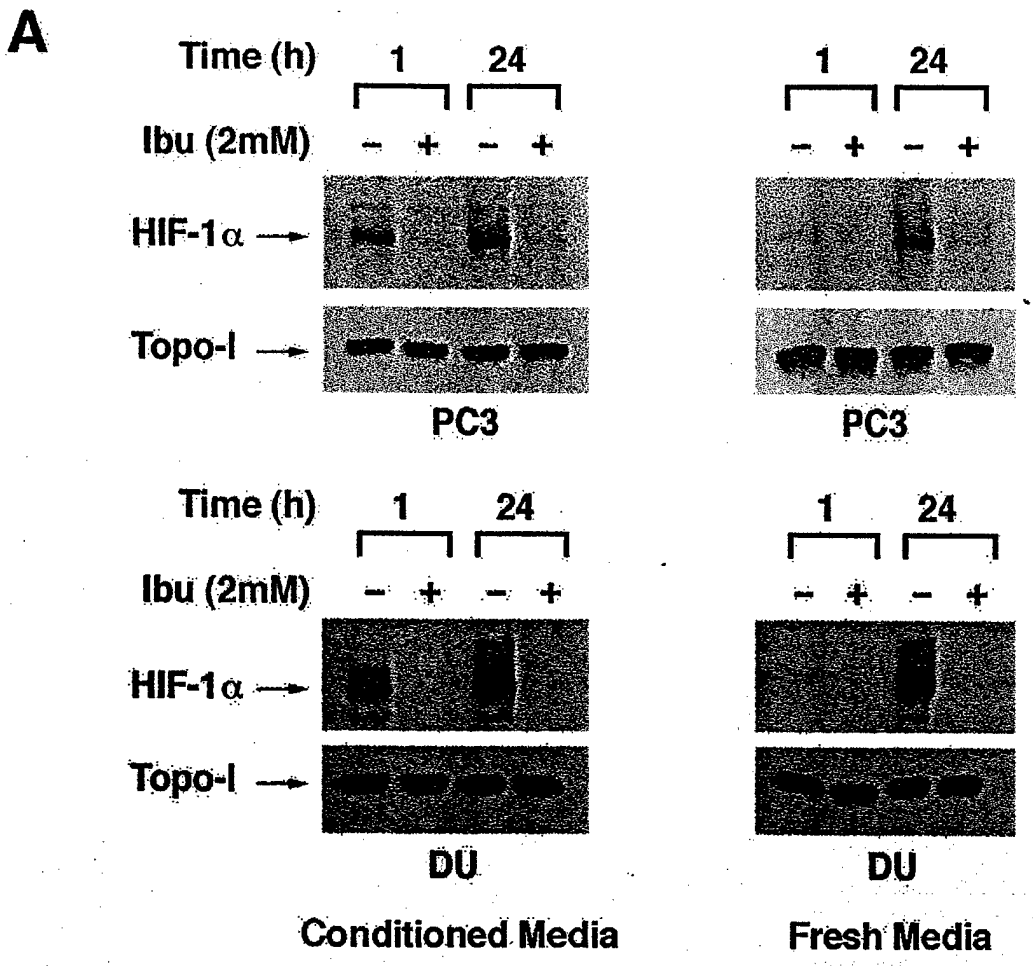
Figure 1


A
Time (h)
1
24
Ibu (2mM)
- + - +
HIF-1α
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PC3
DU
Conditioned Media
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Ibu (mM)
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Figure 2 (A, B)

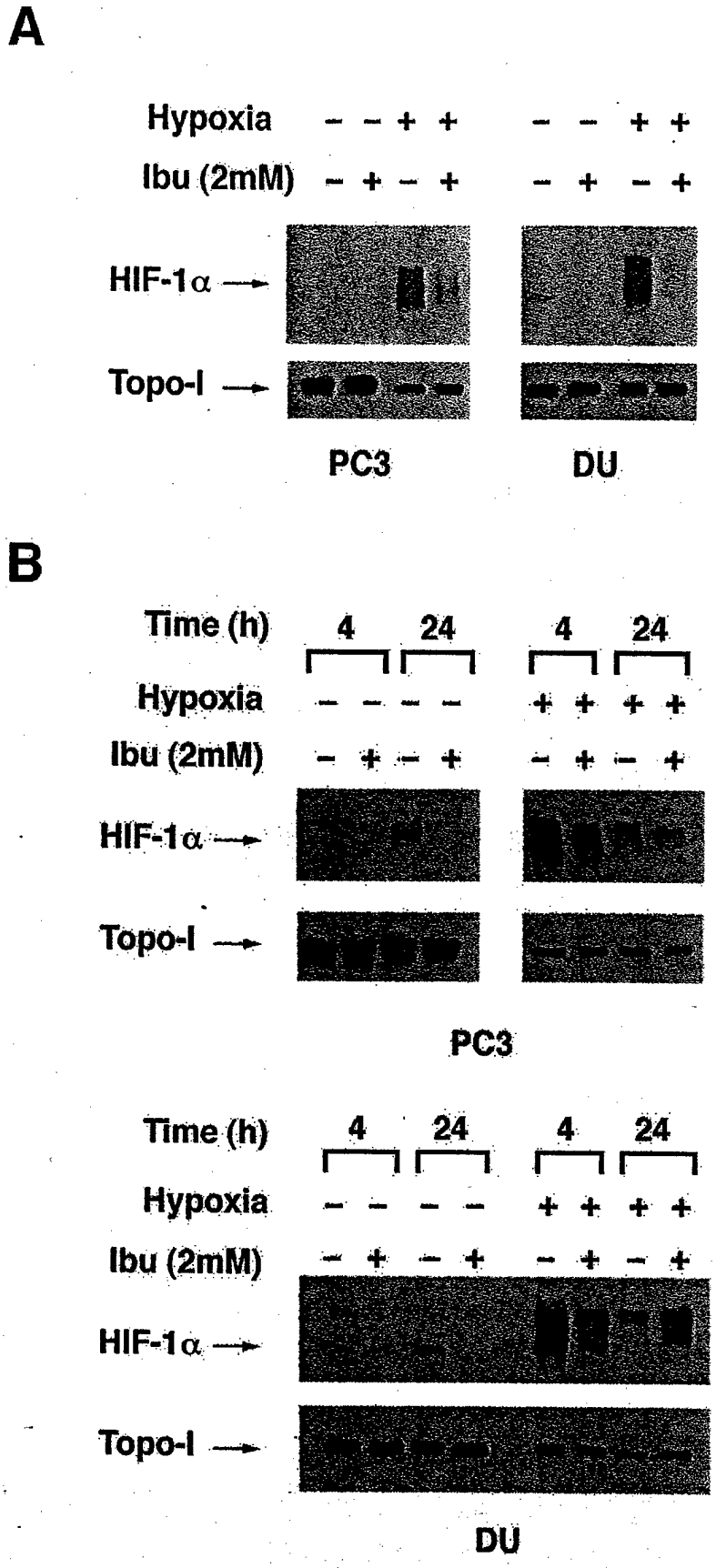
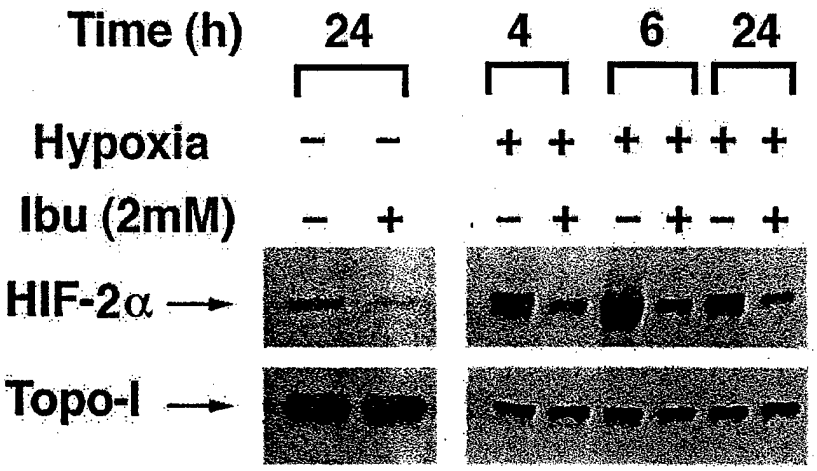
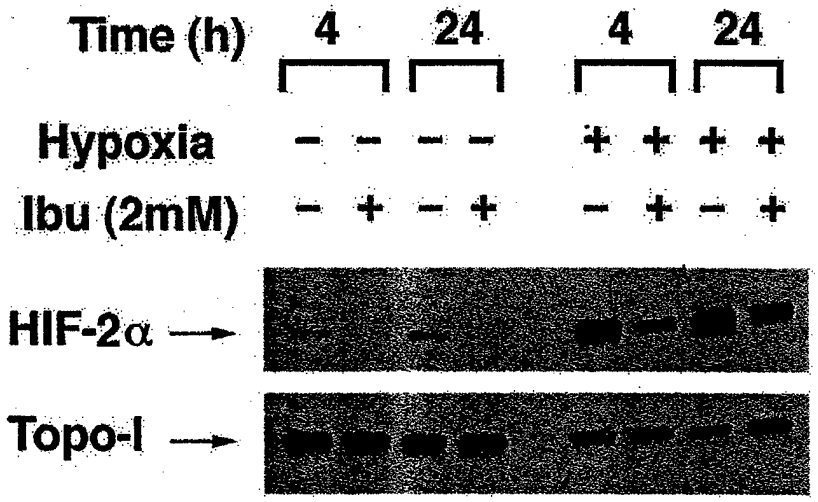

A
Hypoxia
Ibu (2mM)
HIF-1α
Topo-I
PC3
DU
B
Time (h)
4
24
Hypoxia
Ibu (2mM)
HIF-1α
Topo-I
PC3
DU

Figure 2 (C)

C

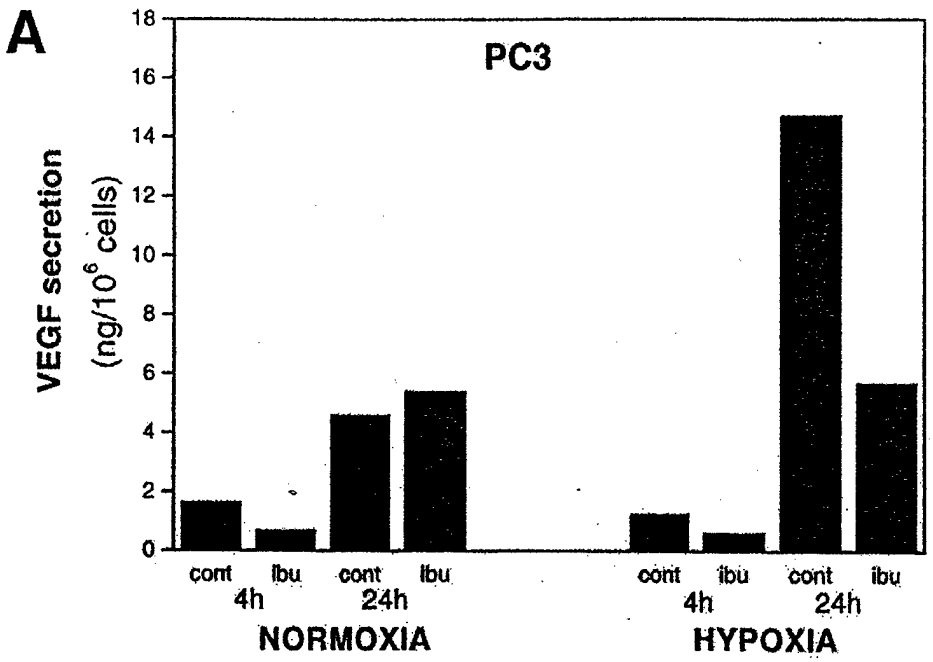


PC3



DU

Figure 3


A
PC3
VEGF secretion
(ng/10^6 cells)
18
16
14
12
10
8
6
4
2
0
cont
Ibu
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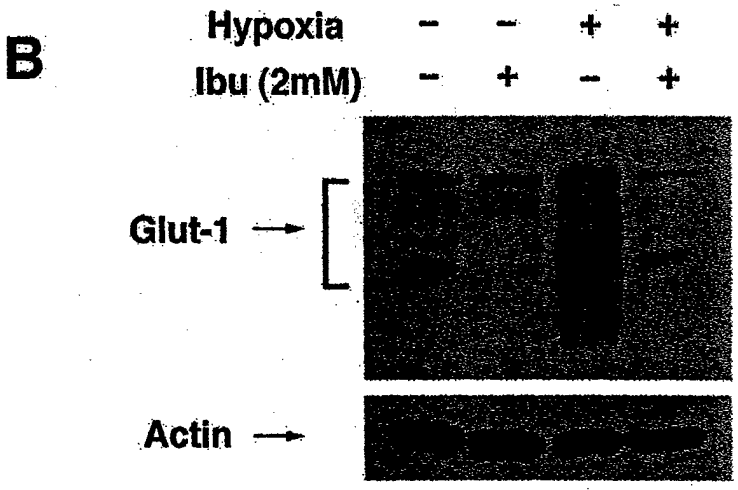
Ibu
4h
24h
NORMOXIA
cont
Ibu
cont
Ibu
4h
24h
HYPOXIA


B
Hypoxia - - + +
Ibu (2mM) - + - +
Glut-1
Actin
PC3

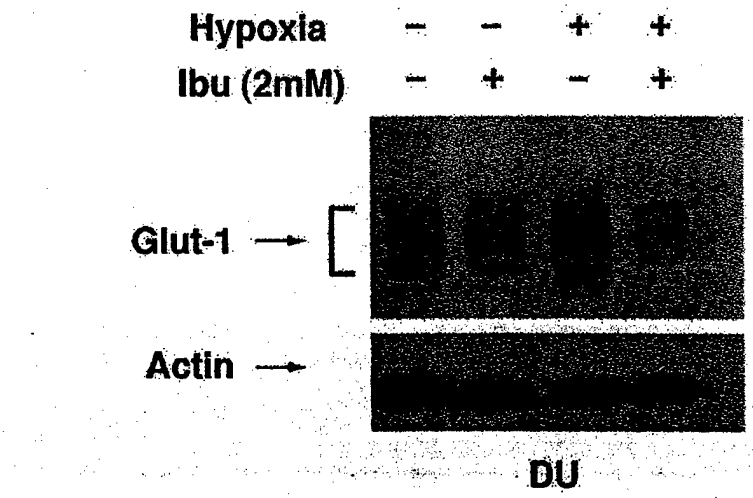
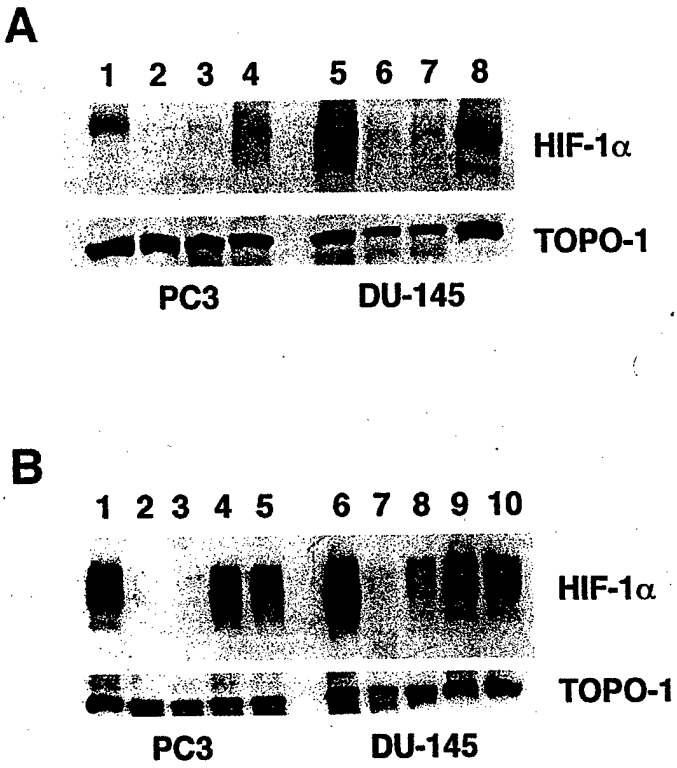

C
Hypoxia - - + +
Ibu (2mM) - + - +
Glut-1
Actin
DU

Figure 4


A
1 2 3 4 5 6 7 8
HIF-1α
TOPO-1
PC3
DU-145
B
1 2 3 4 5 6 7 8 9 10
HIF-1α
TOPO-1
PC3
DU-145

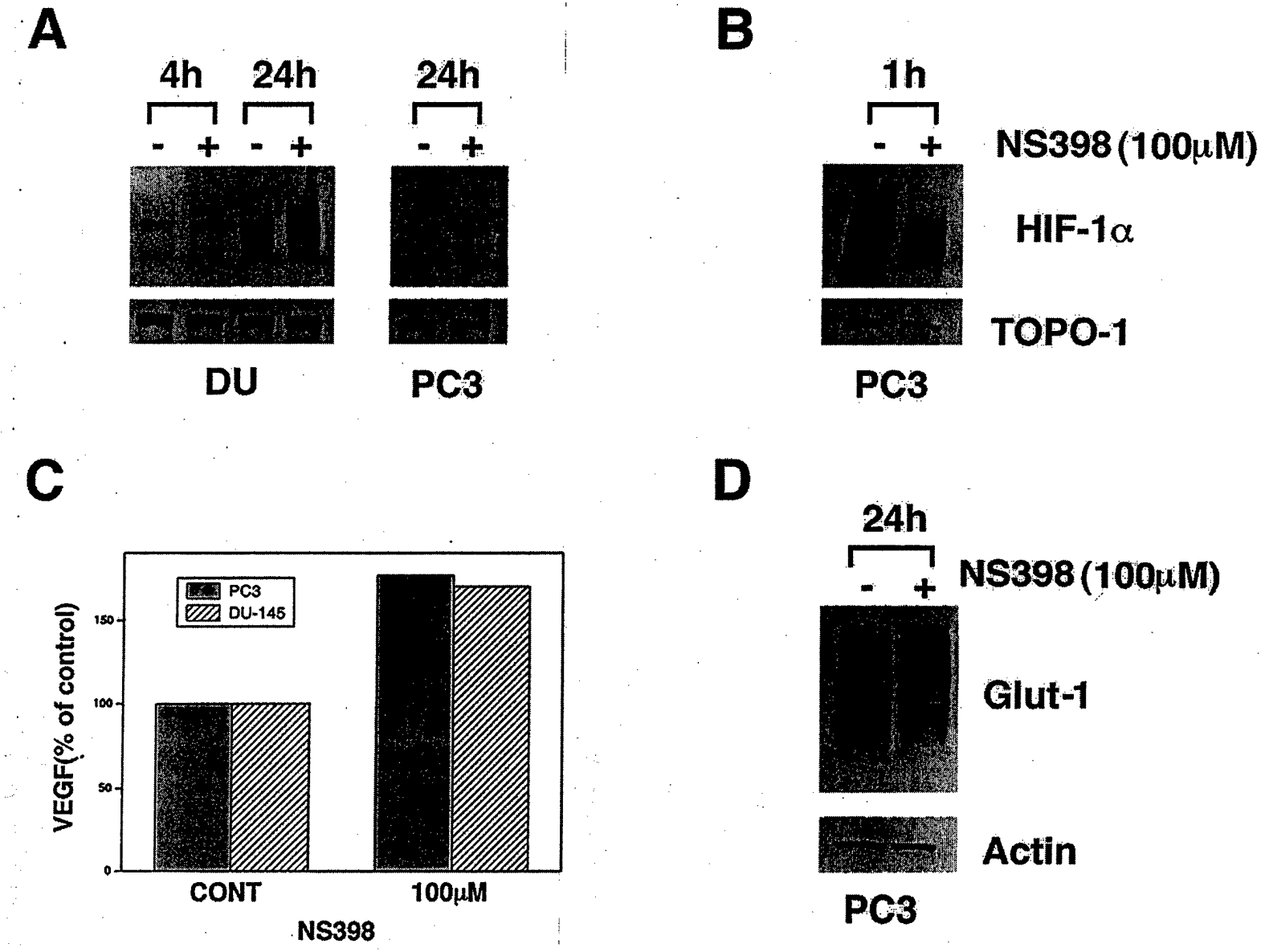
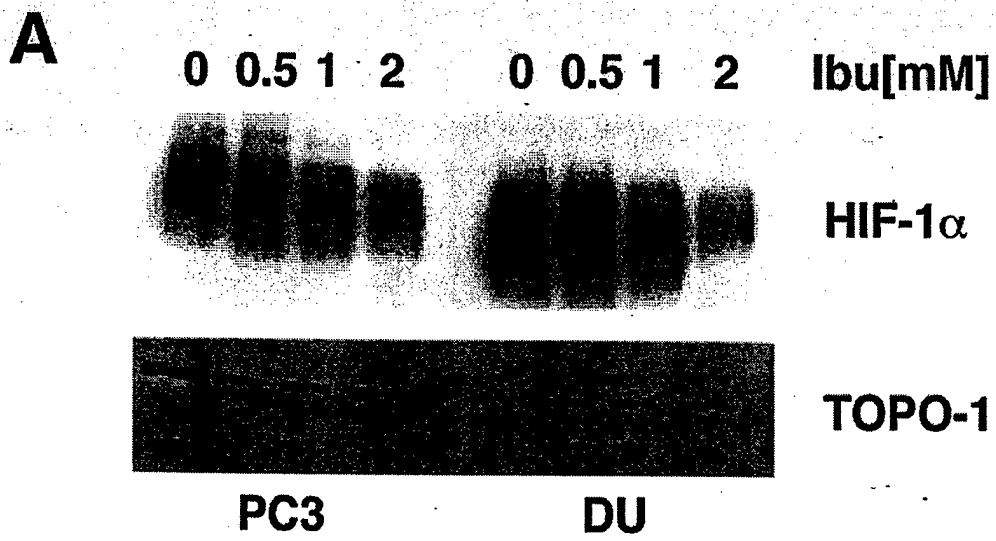
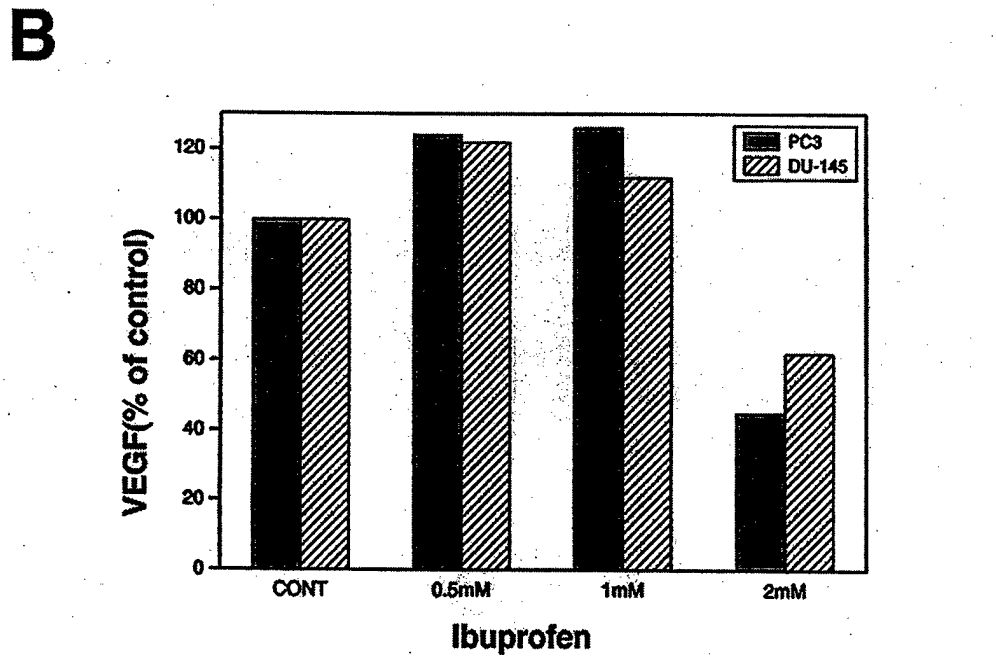

A
4h
24h
24h
- + - +
- +
DU
PC3
B
1h
- +
NS398 (100μM)
HIF-1α
TOPO-1
PC3
C
PC3
DU-145
VEGF(% of control)
150
100
50
0
CONT
100μM
NS398
D
24h
- +
NS398 (100μM)
Glut-1
Actin
PC3

Figure 5

Figure 6


A
0 0.5 1 2 0 0.5 1 2
Ibu[mM]
HIF-1α
TOPO-1
PC3
DU


B
VEGF(% of control)
120
100
80
60
40
20
0
PC3
DU-145
CONT
0.5mM
1mM
2mM
Ibuprofen

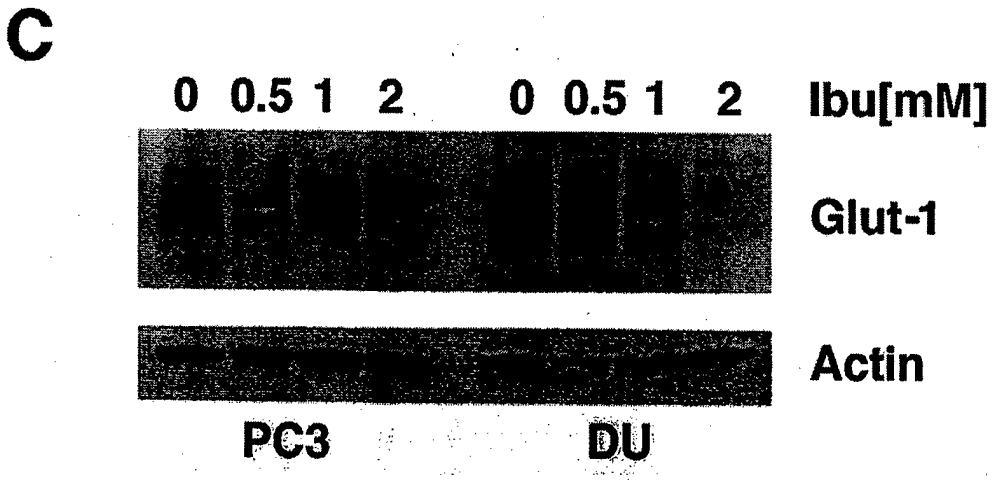

C
0 0.5 1 2 0 0.5 1 2
Ibu[mM]
Glut-1
Actin
PC3
DU

Figure 7